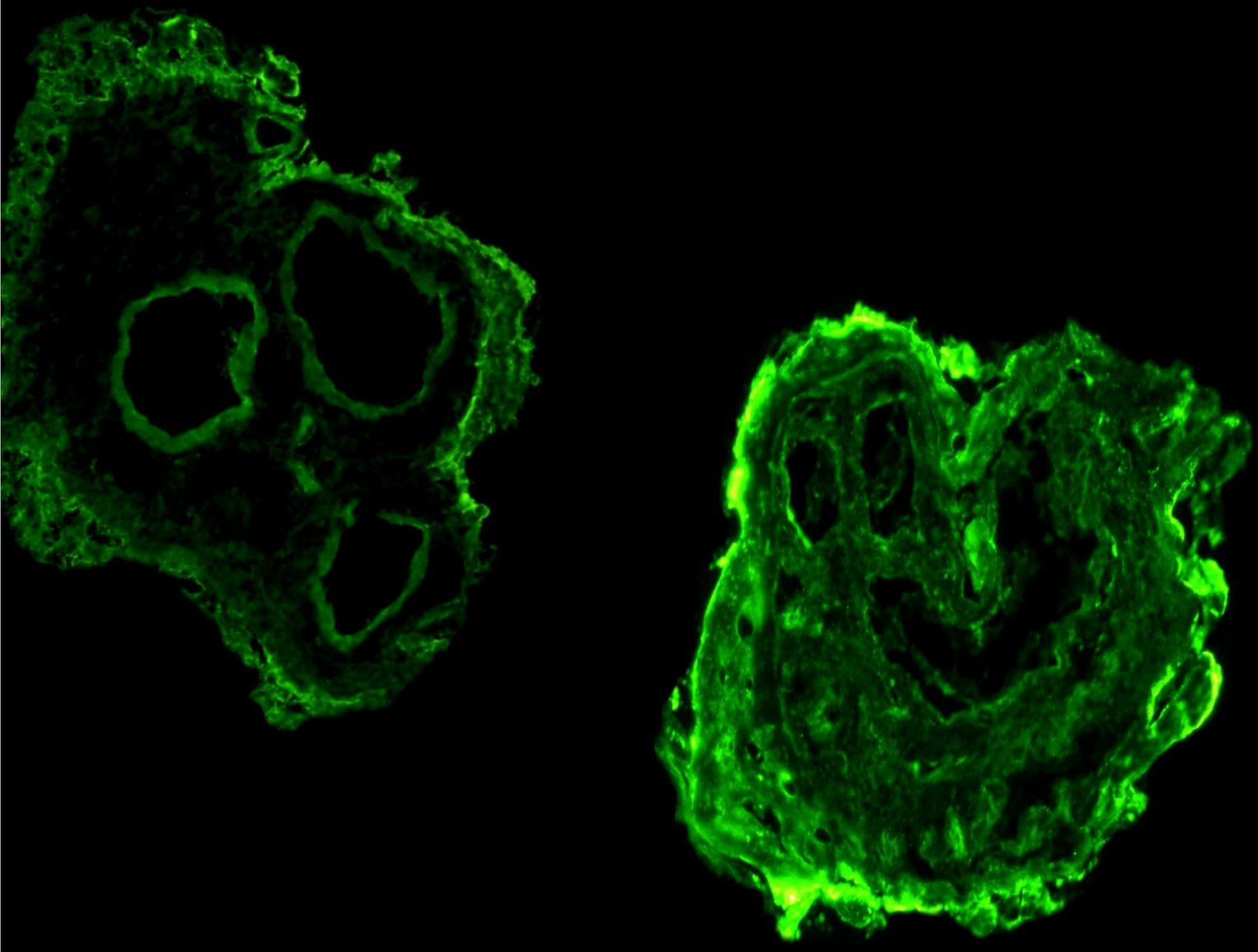
The conundrum of pre-eclampsia – from candidate genes to complement system



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2017

THE CONUNDRUM OF PRE-ECLAMPSIA –
FROM CANDIDATE GENES TO COMPLEMENT SYSTEM

ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Medical Faculty, University of Helsinki, in the Seth Wichmann –hall of Women's Clinic, Haartmaninkatu 2, on the 24th of November 2017 at 12 o'clock noon.

Cover images by the author.
Microscopic photography of cryo sections of the human placenta with immunofluorescent staining.
Left: Early-onset pre-eclamptic pregnancy with CD46/MCP.
Right: Non-pre-eclamptic control pregnancy with C4bp staining.

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"--- observations made in any one era are inevitably limited by the knowledge and the diagnostic aids available at that time and interpretation is coloured by current views."

T.N. Jeffcoate, 1966

To mothers, women of life
To my mother, woman of life and science

CONTENTS

ABSTRACT

The human pregnancy is a unique immunological process. The foetus is tolerated and nourished through the complex yet transient organ, placenta, which bears the genetic fingerprint of the developing baby. The process of placentation is different in humans and large primates in comparison to other mammals. Human placentation involves several waves of invasion, whereby the placenta is deeply attached to layers of the uterine wall, through the mucosal lining of the uterus, the decidua and into the underlying muscle, the myometrium. In order for adequate but not inappropriate depth of placentation to occur, the maternal immune system must be fine-tuned to tolerate yet control the invasive trophoblast cells, which originate in the placenta.

When placentation is successful, the endothelial cells lining the uterine arteries that feed the placenta will be replaced by the endovascular trophoblast cells. Maternal circulation to the placenta is activated at the end of the first trimester. Thereafter the remodelled spiral arteries supply a high-flow, low-resistance placental circulation.

Pre-eclampsia is a common vascular disease of the human pregnancy. Pre-eclampsia, particularly in its severe form, is intrinsically linked to failure of deep-placentation. While pre-eclampsia has long been known to have a familial, i.e. genetic component, the genes contributing to the disease have not been well characterised thus far.

This thesis addresses the question of effects of complement system in pre-eclampsia by comparing pre-eclamptic women to non-pre-eclamptic controls using several methods. Using immunofluorescence and immunohistochemistry, placental tissues were stained for one angiogenic receptor and nine components of the complement system to determine the localisation and extent of complement activation, and on the other hand, complement regulation in placentae from pre-eclamptic and healthy pregnancies. In two candidate gene studies, the variants within coding regions and flanking sequences surrounding exons of a key complement regulator, membrane cofactor-protein (CD46) gene (*MCP*/CD46) and activator, complement component 3 gene (*C3*) were explored in women with a history of severe pre-eclampsia and non-pre-eclamptic controls. The candidate gene studies were done by Sanger sequencing alone (*MCP*), and a combination of Sanger sequencing and sequenom genotyping (*C3*). Finally, using a targeted exomic sequencing approach we studied known candidate genes in pre-eclampsia to identify

low-frequency variants in the Finnish population that may predispose to or protect from pre-eclampsia. We combined pre-eclamptic patients from the FINNPEC (Finnish genetics of pre-eclampsia consortium) and the national FINRISK studies to characterise health consequences of the most important variants that associate to pre-eclampsia.

While we did not find a genetic association to pre-eclampsia in the membrane bound complement regulator, *MCP*, we found three out of forty-three observed single nucleotide polymorphisms to be associated with pre-eclampsia in the *C3.* Furthermore, a section of tight linkage disequilibrium was identified within the *C3* that depending on the sequence context, may predispose to, or protect from pre-eclampsia. We have also provided a description of localisation and presence of the key complement factors in placentae from healthy and pre-eclamptic placentae. Most recently, the first maternal genetic association of the fms related tyrosine kinase 1 gene (*FLT1)* coding for an anti-angiogenetic marker soluble fms-like tyrosine kinase 1 elevated in pre-eclampsia was discovered. Two SNPs within the *FLT1* protect women from not only pre-eclampsia, but also from heart failure in later life.

The work presented in this thesis sheds light to some of the many inherited traits that contribute to the aetiology of pre-eclampsia. The results present a first genetic association to pre-eclampsia in *C3* and they may provide a maternal genetic link between angiogenesis process during pregnancy and heart failure in later life. The crucial effect of the complement system for the successful pregnancy is demonstrated by genetic association as well as comparative localisation of complement components on the pre-eclamptic and healthy placenta tissue.

TIIVISTELMÄ

Raskausaika on ainutlaatuinen immunologinen tapahtuma ihmisen elinkierrossa. Sikiötä ravitsee monimutkainen mutta väliaikainen elin, istukka, joka edustaa perimältään sikiön geneettistä sormenjälkeä. Istukan tehtävä on myös ylläpitää toleranssia äidin immuunipuolustuksessa lapsen kudosten suojelemiseksi. Istukan muodostuminen eli plasentaatio eroaa ihmisten ja muiden suurten kädellisten sekä muiden nisäkkäiden välillä. Ihmisen plasentaatiossa toistuvat istukkasolujen invaasioprosessit varmistavat, että elin kiinnittyy äidin kohdunseinämään limakalvokerrosten alle aina kohdunalaiseen lihaskudokseen asti. Jotta istukan kiinnittyminen ulottuu oikealle tasolle, riittävän syvälle, mutta ei liian syvälle, äidin immuunipuolustuksen pitää olla virittynyt siten, että istukan invasoivia trofoblastisoluja siedetään ja kontrolloidaan samanaikaisesti.

Istukkaa ruokkivat kohdun kierteiset spiraalivaltimot. Plasentaation onnistuessa kohdun valtimoiden sisäseiniä päällystävät endoteelisolut korvaantuvat endovaskulaarisilla trofoblastisoluilla. Äidin verenkierto alkaa ruokkia kasvavaa istukkaa ensimmäisen raskauskolmanneksen lopulla, jolloin muovaantuneet spiraalivaltimot takaavat runsaan mutta matalapaineisen verenkierron istukkaan.

Pre-eklampsia on yleinen monitekijäinen raskaudenaikainen verisuonisairaus. Etenkin vaikea pre-eklampsia liittyy keskeisesti syväplasentaation ilmiöön. Pre-eklampsian tiedetään esiintyvän suvuittain, joten perintötekijöillä on tärkeä osa taudin puhkeamisessa, mutta suurinta osaa pre-eklampsialle altistavista geeneistä ei vielä tunneta.

Väitöskirjassani käsittelen useaa menetelmää hyödyntäen luontaisen immunipuolustuksen komplementtijärjestelmän merkitystä pre-eklampsiataudissa vertailemalla pre-eklamptikkoäitejä ja äitejä, joilla ei ole ollut pre-eklampsiadiagnosia. Istukkakudosleikkeitä värjättiin immunofluoresenssi ja immunohistokemiallisin menetelmin yhtä angiogeneettistä reseptoria ja yhdeksää komplementtitekijää tunnistavilla vasta-aineilla. Tutkimuksen tarkoituksena oli määritellä komplementtiaktivaation sijainti ja laajuus istukkakudoksessa ja toisaalta kuvata komplementilta suojaavien tekijöiden ilmeneminen pre-eklampsiaistukassa ja terveessä istukassa. Kahdessa kandidaattigeenitutkimuksessa kuvattiin koodaavien alueiden ja niitä ympäröivien geenialueiden perimänvaihtelua keskeisessä komplementin

säätelijässä *MCP*/CD46 ja toisaalta aktivaattorissa *C3*. Kandidaattigeenitutkimuksissa vertailtiin vaikeaan pre-eklampsiaan sairastuneiden äitien perimänvaihtelua sellaisten äitien geeneihin, joilla pre-eklampsiadiagnosia ei ole. Kandidaattigeenitutkimukset suoritettiin Sanger-sekvensointimenetelmällä *MCP*-geenitutkimuksessa ja Sanger-sekvensointia ja Sekvenomgenotyyppausta yhdistämällä *C3*-geenitutkimuksessa. Hyödyntämällä kohdennettua eksomisekvensointimenetelmää tutkimme myös muita tunnettuja kandidaattigeenejä pre-eklampsiataudissa tunnistaaksemme harvinaisia perimänvaihtelun muotoja suomalaisessa väestössä, jotka liittyvät pre-eklampsiatautiin. Yhdistimme FINNPEC (Finnish genetics of pre-eclampsia consortium) aineistoon ja FINRISKI-tutkimukseen kirjattujen potilaiden tietoja kuvataksemme pre-eklampsiaan liittyvien perimänvaihteluiden yhteyttä sairastavuuteen taudin jälkeen.

MCP-geenistä ei löydetty pre-eklampsiaan liittyviä perimänvaihtelun muotoja, mutta *C3*-geenissä kuvatusta neljästäkymmenestä kolmesta pistemutaatiosta kolmen todettiin liittyvän pre-eklampsiaan. Lisäksi *C3*-geenin keskivaiheilla kuvattiin sekvenssialue, jossa on vahva kytkentäepätasapaino. Riippuen alueen emäsjärjestyksestä, sen todettiin joko altistavan tai suojaavan pre-eklampsiataudilta. Viimeisimpänä kuvattiin ensimmäistä kertaa äidin perinnöllinen suojaava tekijä *FLT1*-geenistä, joka koodaa verisuonien muodostukseen liittyvää tekijää sFlt-1, jonka lisääntyneen määrän verenkierrossa tiedetään liittyvän pre-eklampsiariskiin. Kahden pistemutaation *FLT1*-geenissä todettiin suojaavan paitsi pre-eklampsialta, myös sydämen vajaatoiminnalta.

Väitöskirjassani esittelemä tutkimus lisää ymmärrystä monista perintötekijöistä, jotka liittyvät pre-eklampsiataudin kehittymiseen. Tuloksissa kuvataan ensimmäistä kertaa perinnöllinen yhteys pre-eklampsiataudin ja *C3* geenin välillä. Toisaalta äidin verisuontenmuodostusta säätelevän tekijän osalta osoitetaan perinnöllinen yhteys raskaudenaikaisen terveyden ja vähentyneen sydämen vajaatoimintaan sairastuvuuden välillä. Komplementtijärjestelmän keskeinen merkitys terveessä raskaudessa käy ilmi sekä perintötekijöiden kautta että kudosvärjäystutkimuksen keinoin pre-eklampsiaraskauksien ja terveiden raskauksien istukoita vertailemalla.

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*equal contribution

ABBREVIATIONS

ACVR1	activin A receptor type 1 gene
ACVR1C	activin A receptor type 1C gene
ADAM12	ADAM metallopeptidase domain 12 gene
ADAM28	ADAM metallopeptidase domain 28 gene
ANGPT4	angiopoietin 4 gene
ANGPTL1	angiopoietin-like protein 1 gene
AP	alternative pathway
C	complement system
C1q	complement component 1, q subcomponent
C2	complement component 2
C3/*C3*	complement component 3, protein/gene
C4/*C4*	complement component 4, types A and B, protein/gene
C4bp	C4b binding protein
C5	complement component 5
CCP	complement control protein domain
CD	cluster of differentiation
CFB	complement factor B gene
cffDNA	cell-free foetal DNA
CFH	complement factor H gene
CFP	complement factor properdin gene
CLU	clusterin protein
CO	carbon monoxide
corin/*CORIN*	corin, serine peptidase, protein/gene
CRP	C-reactive protein
Crry	complement receptor related protein (*Mus musculus*)
CP	classical pathway
CUB	complement C1r/C1s, Uegf, Bmp1 - domain
CVD	cardiovascular disease
DAPI	4',6-diamidino-2-phenylindole stain
DF	degrees of freedom
DNA	deoxyribonucleic acid
E	endothelial cells
EHD3	EH domain containing 3 gene
EOPE	early-onset pre-eclampsia
EVT	extra-villous trophoblast
EvTC	endovascular trophoblast cells
F_A	frequency_affected
FB/*FB*	factor B protein/gene

FCN2	ficolin 2 gene
FCN3	ficolin 3 gene
FD	factor D
FH	factor H
F_U	frequency_unaffected
FI	factor I
FIMM	Institute for Molecular Medicine Finland
GH	gestational hypertension
GPR98	adhesion g protein-coupled receptor V1 gene
FLT1	fms related tyrosine kinase 1 (VEGFR1) gene
FLT4	fms related tyrosine kinase 4
HC	hofbauer cells
HE	haematoxylin and eosin stain
HELLP	haemolysis, elevated liver enzymes, low platelets
HF	heart failure
HIV-1	human immunodeficiency virus 1
HSF	human splicing finder
IL-10/*IL10*	interleukin 10, protein/gene
INHA	inhibin alpha subunit gene
INHBA-AS1	inhibin beta A subunit antisense RNA 1 gene
INHBE	inhibin beta E subunit gene
ITC	interstitial trophoblast cells
ISSHP	the international society for the study of hypertension in pregnancy
IUGR	intra-uterine growth restriction
JAG1	jagged 1 gene
KDR	kinase insert domain receptor gene
LCT	lactase gene
LOF	loss of function
LOPE	late-onset pre-eclampsia
LP	lectin pathway
M	macrophage
mAb	monoclonal antibody
MAC	membrane attack complex
MAF	minor allele frequency
MASP1/*MASP1*	mannan-binding lectin serine peptidase 1 protein/gene
MASP2	mannan-binding lectin serine peptidase 2 gene
MBL2	mannose-binding lectin 2 gene
MCP/*CD46*	membrane cofactor-protein protein/gene
NCBI	national center for biotechnology information
NET	neutrophil extracellular traps
NK	natural killer cell
NOS3/*NOS3*	nitric oxide synthase 3, protein/gene

pAb	polyclonal antibody
PCR	polymerase chain-reaction
PlGF	placenta growth factor
POC	parent-offspring conflict
pp13	galectin 13
PZP	PZP, alpha-2-macroglobulin like, pregnancy zone protein gene
qPCR	quantitative polymerase chain-reaction
RCA	regulators of complement activation gene cluster (chr 1)
RNA	ribonucleic acid
RT	room temperature
RUPP	reduced uterine perfusion pressure rat model
SD	standard deviation
SERPING1	serpin peptidase inhibitor, member1 gene
sEng	soluble endoglin
sFlt1	soluble fms-like tyrosine kinase 1
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
T	T-cell
TC	trophoblast column
TED	thio-ester containing domain
TEK	TEK receptor tyrosine kinase gene
TGFBR2	transforming growth factor beta receptor 2 gene
THBD	thrombomodulin gene
THBS1	thrombospondin 1 gene
THBS3	thrombospondin 3 gene
THBS4	thrombospondin 4 gene
TMA	thrombotic microangiopathy
TP	terminal pathway
TREX1	three prime repair exonuclease 1 gene
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor 1 (*FLT1*)
VEP	variant effect predictor
VTN	vitronectin

1. INTRODUCTION

The integrity of human organism, as any living being, is maintained and ensured by a functional immune system. The purpose of immune system is to discriminate between self- and non-self structures, to provide defensive mechanisms against pathogens, and to maintain health of the organism by clean up of debris and unwanted articles. The human immune system may be divided into innate and adaptive arms. Innate immune system is the evolutionarily ancient first line of defence, which recognises unwanted structures and initiates immune response. Adaptive immune system develops throughout the course of the individual's life to provide specific defence against a variety of pathogens.

The second classification that is widely used to describe the immune system is the division between humoral and cellular immunity. The innate and adaptive immune systems have components from both categories. Humoral immunity is comprised of activating or inhibiting proteins that circulate in the plasma and serum or are formed in tissues to activate the immune system. Cellular immunity refers to cell-mediated immunity, such as recognition of antigenic peptides, and activation of cells in the adaptive immune system by antigen presenting cells.

Pregnancy presents an exceptional challenge to the immune system. The developing foetus and placenta are genetic allografts that may be recognised as foreign by the maternal immune system. For this reason, numerous immunosuppressive processes take place in the maternal immune system during pregnancy to protect the developing foetus. Breakdown of any of these protective mechanisms may result in adverse pregnancy outcomes. In order for placentation to be successful, the foetal cells must evade the maternal immune system to survive and invade into the maternal tissues. The purpose of trophoblast invasion is to ensure sufficient blood flow by anchoring the placenta deep into the maternal tissue and to improve maternal blood flow to the uterus in a process known as spiral artery remodelling.

In this thesis work, I have explored the maternal innate humoral immune system in pre-eclampsia, a common vascular disease of the pregnancy. I also searched for genetic associations among known candidate genes including those involved in angiogenesis, a key process of placental development.

Complement system is an ancient part of innate immunity, which consists of cell surface bound and freely circulating proteins that interact in a cascade of activation and regulation. Complement system has the capacity to discriminate between self and non-self cells and particles and thereby maintain tolerance or activate adaptive immunity. Complement activation can lead to inflammation, cell death, and tissue destruction. The component C3 is at the core of the complement system. It can be activated by three different pathways of the complement system. C3 can also become spontaneously activated in the human serum.

To protect own tissues from complement-mediated destruction and death, interaction between tissues and the complement system must be carefully regulated. While soluble inhibitors are important in controlling complement activation, the cells of the human body are also protected by surface-bound regulators of the complement system. One of these potent regulators is the membrane co-factor protein (MCP, cluster of differentiation 46/CD46). The gene coding for this protein, *MCP* has previously been implicated in pre-eclampsia. As a part of this study, we explored the association of genetic polymorphisms of *MCP* to pre-eclampsia.

Complement components can be visualised in the placenta. Of particular interest is the possible deposition, coating, and localisation of complement activators and regulators on the maternal-foetal interface, the syncytiotrohoblast cell layer, which circumferentially lines the foetal tissues facing the maternal blood flow in the intervillous space of the placenta.

In this thesis, I will discuss the role of the complement system in pre-eclampsia. I hypothesise that in a subset of pre-eclamptic patients, particularly those with the severe or early-onset disease, over-activation or lack of regulation of the complement system may predispose these patients to pre-eclampsia. Pre-eclampsia being a complex genetic disease, heritable predisposition within genes coding for components of the complement system are mostly studied. To address the multifactorial aspect of the disease, candidate genes with previously suggested association to pre-eclampsia from other pathways are also studied. While genetic association studies of the maternal complement system and other candidate genes are at the core of the study, foetal complement system is studied in immunohistochemistry, and *C4* copy number variations in both foetal and maternal samples are also assessed.

2. REVIEW OF THE LITERATURE

2.1 Pre-eclampsia - disease of many symptoms and few explanations

Pre-eclampsia was first described by the Greek philosopher and surgeon Hippocrates, who described the grand-mal seizures inflicting a previously healthy expecting mother as surprising as lightning. The diagnostic separation of pre-eclampsia from hysteria or epilepsy was established throughout the 16th century (Schlembach 2003).

"--- we now have all the essential facts bearing on the aetiology of eclampsia, and that they resemble the pieces of a jig-saw puzzle and only need to be assembled in proper sequence to provide an acceptable explanation of eclampsia, including its onset two or three days after delivery."

F.J. Browne, 1958

Pre-eclampsia was branded as a disease of theories over half a century ago (Jeffcoate 1966). The label has stood the test of time (Figure 1) (Broughton Pipkin & Rubin 1994, Mignini *et al.* 2006, Widmer *et al.* 2007, Dennis & Castro 2014). Pre-eclampsia is a complex multifactorial disease with a variable spectrum of symptoms. The key diagnostic criteria used worldwide are new-onset proteinuria combined with elevated blood pressure during the second half of the pregnancy. Subjective symptoms that may or may not appear include headaches, vision impairment, nausea, oedema, and abdominal pain.

The systemic nature and aetiological contribution of the placenta to the disease was identified already in the beginning of the last century (Holland 1909). Nutritional and microbial triggers were previously at the centre of the research, and immunological mechanisms and hypertensive predisposition have been under investigation since early days. Familial predisposition to pre-eclampsia was recognised early (Chesley *et al.* 1968). With the development of molecular biomedical research, genetic studies have been most abundant in recent years.

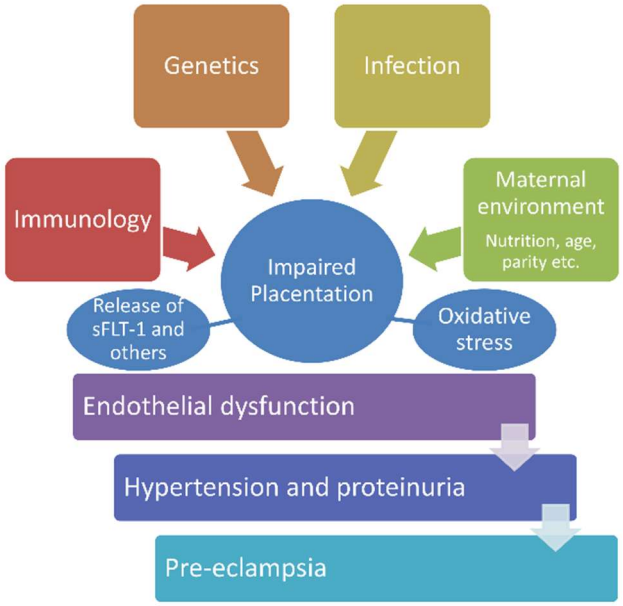

Figure 1. Theories of pre-eclampsia. While the underlying cause is debated on, in majority of the cases the underlying mechanistic problem in pre-eclampsia is inadequate trophoblast invasion resulting in impaired placentation. Together with other factors, the result is systemic endothelial dysfunction, which most often manifests as hypertension and proteinuria during the second half of the pregnancy resulting in the diagnosis of pre-eclampsia.

2.2 Burden of pre-eclampsia

Pre-eclampsia is a serious complication of human pregnancy. It affects 3–5% of pregnancies in all ethnic groups. The onset and clinical course of pre-eclampsia is unpredictable. Pre-eclampsia is a leading cause of pregnancy related morbidity and mortality worldwide. The burden of pre-eclampsia on the healthcare system is mainly focused at the maternal and foetal mortality in developing countries and at the complications related to prematurity of the infants born from pre-eclamptic pregnancies in the Western world. Pre-eclampsia increases perinatal mortality five-fold attributing to 10-19% of maternal deaths worldwide

(Lain & Roberts 2002, Duley 1992, World Health Organization 2005). In the developing countries pre-eclampsia leads to approximately 50 000 maternal deaths annually (World Health Organization 2005). It can lead to multi-organ dysfunction and, rarely, to a life-threatening convulsive condition, eclampsia (Roberts *et al.* 2003). Presently, delivery of the placenta remains the only cure for pre-eclampsia. The aetiology of pre-eclampsia is still largely unknown. Key features of pre-eclampsia include vascular impairment, systemic endothelial disturbance, and placental disruption and dysfunction.

2.3 Diagnosis and definition

Pre-eclampsia is a complex multifactorial disease with a variable spectrum of symptoms. The key criteria used worldwide are new-onset proteinuria combined with elevated blood pressure during the second half of the pregnancy, or new onset pre-eclampsia-associated findings in the absence of proteinuria (American College of Obstetricians and Gynecologists & Task Force on Hypertension in Pregnancy 2013, Tranquilli *et al.* 2014). Pre-eclampsia-associated findings include thrombocytopenia, renal insufficiency, impaired liver function, pulmonary oedema and cerebral or visual symptoms (American College of Obstetricians and Gynecologists & Task Force on Hypertension in Pregnancy 2013). The variability of symptoms reflects the likelihood that pre-eclampsia is not a single disease, rather resembling a syndrome (Roberts & Escudero 2012, Myatt & Roberts 2015). For the benefit of research, further classification of pre-eclampsia patients is commonly performed according to the specific symptoms and timeline of the disease.

According to severity of symptoms and onset of the disease or delivery time, pre-eclampsia may be classified into further sub-phenotypes of severe or non-severe pre-eclampsia and early or late onset pre-eclampsia. After the studies in this thesis were initiated, the International Society for the Study of Hypertension in Pregnancy (ISSHP) released new guidelines for diagnosing pre-eclampsia. According to the ISSHP 2014 guidelines, pre-eclampsia may be diagnosed in a hypertensive patient in absence of proteinuria, if one or more criteria are met from a list including maternal organ dysfunction (elevated liver enzymes, haematological complications, renal insufficiency, and neurological symptoms) and uteroplacental dysfunction (Kallela *et al.* 2016, Tranquilli *et al.* 2014). Furthermore, according to the ISSHP 2014

recommendations, due to the unpredictability of the disease, in clinical practice all patients should be treated as potentially severe pre-eclamptics (Tranquilli *et al.* 2014). This study treated disease onset before 34 weeks of pregnancy as early-onset pre-eclampsia, which is in accordance to the ISSHP 2014 recommendations (Tranquilli *et al.* 2014).

2.3.1 Vascular impairment

Increased resistance in uterine arteries can be detected in Doppler ultrasound already in the early second trimester of pregnancy in women who will develop pre-eclampsia later in their pregnancy (Harrington *et al.* 1996). Vascular impairment caused by pre-eclampsia is mirrored in later life by increased risk of cardiovascular disease (CVD) (Irgens *et al.* 2001, Sattar & Greer 2002, Bellamy *et al.* 2007).

2.3.2 Systemic endothelial disturbance

Endothelial disturbance results in disruption of the blood vessel barrier as well as altered function of the endothelial cells. Several of the typical findings in pre-eclampsia are due to endothelial dysfunction including damage of the glomeruli, activation of the coagulation cascade, and oedema (Roberts *et al.* 1989).

Systemic endothelial disturbance is reflected in some of the more uncommon end-points of severe pre-eclampsia. It is poorly understood which pre-eclampsia sufferers will develop life-threatening complications. Such complications may affect the central nervous system resulting in seizures and eclampsia, or the liver resulting in diagnosis of haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome. Rarely, function of the heart and lungs may also be compromised as a result of complicated pre-eclampsia.

2.3.3 Placental disruption

Placental disruption and dysfunction is particularly associated with the early-onset and severe pre-eclampsia phenotypes. In 20% of early-onset cases inadequate trophoblast invasion is associated with hypoxia (Huppertz 2008). Notably, the pre-eclamptic placenta has been observed to be immature in its structure. In early-onset pre-eclampsia, inadequate trophoblast invasion in the early stages of placentation leads to incomplete spiral artery remodelling causing poor placental development and oxidative stress in later pregnancy (Figure 2.)

(Khodzhaeva *et al.* 2015). Inefficiency of trophoblast invasion to myometrium and resulting inadequate spiral artery remodelling is not unique to pre-eclampsia, but it is most common in severe pre-eclampsia (Meekins *et al.* 1994).

Furthermore, inadequate remodelling of the spiral arteries creates turbulent and constrained blood flow to the intervillous space (Roth *et al.* 2017). The irregular blood flow increases not only the physical strain on the placental tissue causing shedding of the syncytiotrophoblast and the subcellular material within, but also the deficiency of nutrient supply to the foetus (Pijnenborg *et al.* 2006).

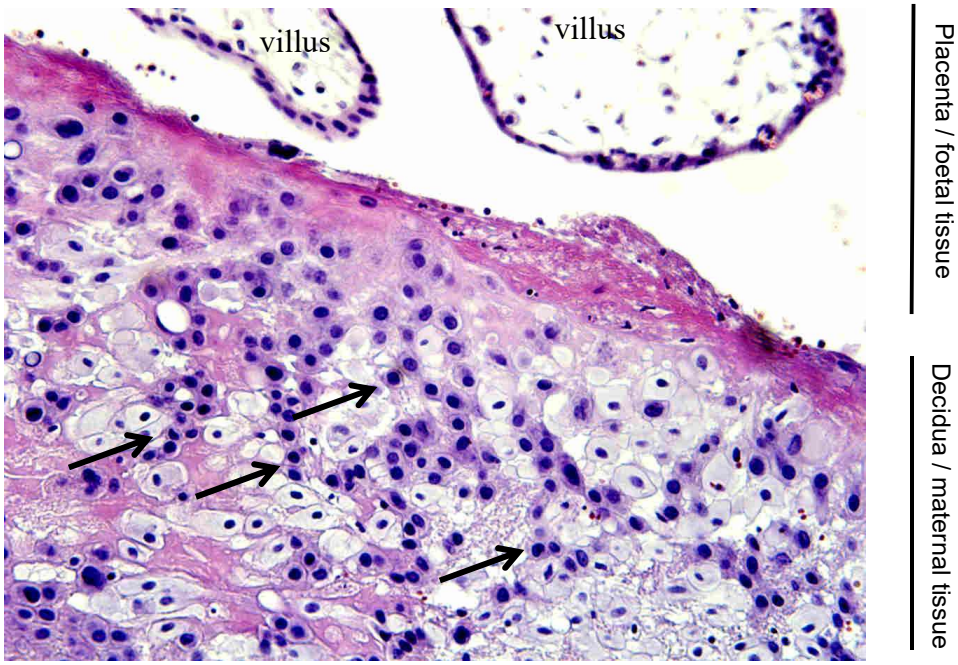

Figure 2. A microscopy image (magnification 200x) of HE stained placenta at week 19 shows extravillous trophoblasts (EVT) migrating into the decidua invading the maternal tissue. The EVT are pointed out with black arrows and they have a round to oval dark nuclei and they penetrate between the round lighter shaded decidual cells. Similar EVT migration trails are seen in the decidua of placentae at week 8 in the event of the first invasive phase. Courtesy of B. Huppertz, University of Graz, Austria keeping the Kaufmann series of placental development slides.

2.3.4 Development of the disease

The main phenomena listed above constitute pre-eclampsia and interact to aggravate the disease. Vascular impairment will promote placental dysfunction by increasing physical shear and tear to the placental tissue due to turbulent and forceful blood flow from the constricted spiral uteral arteries (Figure 3) (Roth *et al.* 2017). Disruption of the placental tissue releases subcellular material such as cell-free foetal DNA (cffDNA) as well as syncytial fragments into the maternal blood (Roth *et al.* 2017). Excess foetal material released into the maternal blood stream may contribute to systemic endothelial dysfunction and inflammation and predict the onset of severe complications such as HELLP (Swinkels *et al.* 2002).

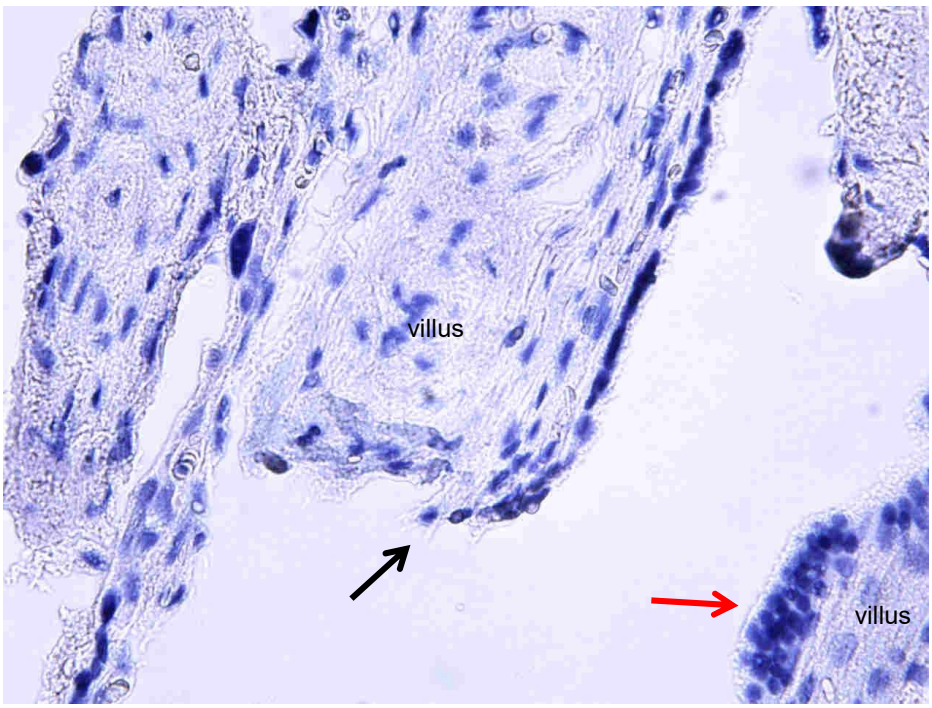

Figure 3. Early-onset pre-eclamptic placenta (wk 34) showing the typical syncytial disruption, where the syncytium is no longer even and encapsulating the villus circumferentially but instead the syncytium is shredded and bursting (black arrow) to release sub-cellular material into the maternal circulation. Another typical feature of the pre-eclamptic syncytium is the syncytial knot (red arrow) that is a cluster of nuclei that may be shed into the intravillous space. DAPI (4',6-diamidino-2-phenylindole) staining, magnification 400x.

2.4 *Evolutionary perspective - human predisposition to pre-eclampsia*

2.4.1 Evolution of placentation

Pre-eclampsia is a disease that only affects humans and larger primates (Crosley *et al.* 2013). It is known that among non-human mammals, only great apes (gibbon, gorilla, chimpanzee, orang-utan) share some of the key features of human placentation, such as varying depth of trophoblast invasion, the consequent spiral artery remodelling, and the presence of intravillous space (Carter & Pijnenborg 2011, Gundling & Wildman 2015). Studying the shared mechanisms of the deep placentation among the primates may provide novel information about pregnancy disorders, because many severe obstetric pathologies are related to an erroneous level of placentation depth (Brosens *et al.* 2011).

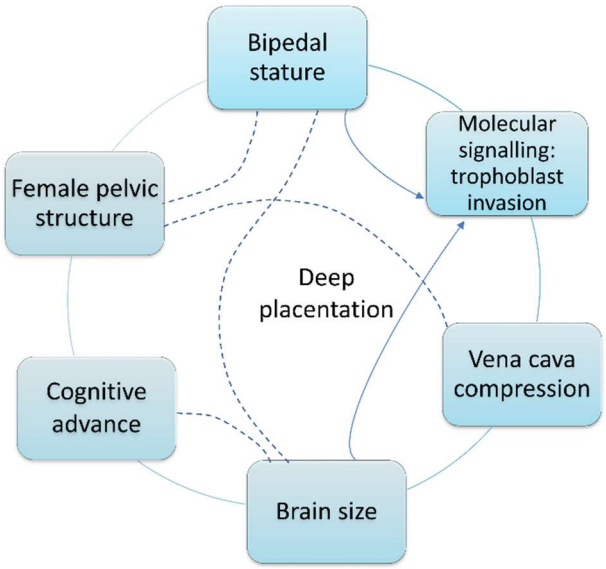

Figure 4. Interactions between processes contributing to deep placentation

It has been suggested that the development of bipedal posture in hominid evolution had several consequences to the reproductive process resulting ultimately in deep placentation (Figure 4.). The upright stature resulted in modification of the female pelvis to initially enable the upright stance and later to accommodate for the increased brain size of the infant through unique rotating birthing mechanism (Fischer & Mitteroecker 2015, Rosenberg & Trevathan 2007). The increase in cognitive ability resulting from larger brain size led to improved foraging efficiency of early hominids. Adequate foraging efficiency allowed for the increase in nutrient transfer to the foetus, which is enabled by deep placentation.

The evolutionarily unique placentation process is mirrored in the molecular fingerprint of the placenta as well as the genetic constitution on which the process of placentation develops (Crosley *et al.* 2013, Elliot & Crespi 2015). Only hominids (orang-utan, gorilla, chimpanzee and human) have galectin cluster in chromosome 19. Furthermore, galectin-13 (pp13) is unique to Old World monkeys and apes, which have intense spiral artery remodelling by the invasive trophoblast (Pijnenborg *et al.* 2011a, Pijnenborg *et al.* 2011b). Since pp13 may be a novel player in introducing and maintaining maternal tolerance to the semi-allogeneic foetus, it is a possible link between deep placentation, pre-eclampsia and immunogenetic complexity (Than *et al.* 2014, Pijnenborg *et al.* 2011a).

There is ample evidence that the depth and efficiency of placentation are at a co-evolutionary deadlock (Wells 2015). This phenomenon is known as the Red Queen hypothesis of an evolutionary arms race (Pijnenborg *et al.* 2008). The Red Queen hypothesis states, that evolutionary arms race leads to an endpoint, where due to competing improvement, each counterpart succeeds as well as they did in the beginning (Van Valen 1973). The conflicting selection pressures behind the co-evolutionary process of placentation are derived from the foetus aspiring for a deeper placentation and more nutrients to be transferred and the mother keeping her physiological homeostasis in check and restricting the amount of nutrients invested in rearing offspring.

2.4.2 Pre-eclampsia – an evolutionary paradigm

Pre-eclampsia affects all human populations worldwide with comparable frequency, usually approximately 3% of pregnancies (Roberts *et al.* 2011). However, Vietnam and Niger have an exceptionally low rate of pre-eclampsia of approximately 1.1% (Bilano *et al.* 2014). In comparison, African-American and certain Latin American populations have at least double the incidence, when compared to populations of European origin (Bilano *et al.* 2014, Breathett *et al.* 2014). While some of the variation can be explained by availability of antenatal care, over- or under- reporting of the cases, or the obesity epidemic, there seem to be underlying differences in pre-eclampsia incidence between populations at the global level.

Pre-eclampsia belongs unquestionably to the leading causes of reproductive failure in humans. Therefore, it is surprising that in the quest to understand and describe the disease pathogenesis, evolutionary causes and consequences have received relatively limited attention. Heritability is the ratio of genetic variance to the sum of genetic and

environmental variance noted as h^2, which can have a value between 0 (non-heritable trait) to 1 (100% heritable trait). By the rules of natural selection, a heritable ($h^2 > 0.55$) predicament leading to decreased reproductive success, such as pre-eclampsia, is expected to disappear from the population, unless a counteracting balancing selection process is at work (Cnattingius *et al.* 2004, Brown *et al.* 2013).

A growing body of evidence suggests that pre-eclampsia associates with vascular and metabolic diseases that typically manifest later in life (Ramsay *et al.* 2003, Christensen *et al.* 2017, Bokslag *et al.* 2017). While epidemiologically relevant, these disease associations after the prime reproductive age offer little in terms of evolutionary evidence for the development and maintenance of pre-eclampsia as a constant phenomenon in the human population.

2.4.3 Human reproduction – an evolutionary challenge

Humans typically have a very tight fit between the maternal pelvic form and size and the size of the baby. At the core of the evolutionary seesaw of pregnancy is the size of the baby (Figure 5.). While maintaining a viable robust offspring, it is advantageous for the mother to produce as small offspring as possible, due to larger metabolic demands and increased risks during childbirth related to rearing larger infants (Fischer & Mitteroecker 2015). In contrast, it is advantageous for the offspring to extract as much nutrients as possible through the placenta while still securing a safe birth. Large birth size will store nutrients and support neurological development. This parent-offspring conflict (POC) is reflected in the expression of paternally and maternally imprinted genes during pregnancy. Recent theories suggest that the size of the baby is mostly influenced by the environmental factors, i.e. the metabolic signalling from the mother in order to compensate for intergenerational shifts in pelvic structure due to changes in ecological conditions and nutritional status (Wells 2015).

Reduced size of the baby combined with signalling to induce elevated maternal blood pressure may be an evolutionary response to ischemic uteroplacental circulation (Espinoza 2012). Indeed, POC has been shown to act as a selection pressure via timing of pregnancy induced hypertension or gestational hypertension (GH) (Hollegaard *et al.* 2013). Early onset GH is beneficial for the offspring reducing early mortality and risks in later life, while GH during later trimesters is deleterious increasing these risks to the level of co-occurrence of GH with pre-eclampsia (Hollegaard *et al.* 2013).

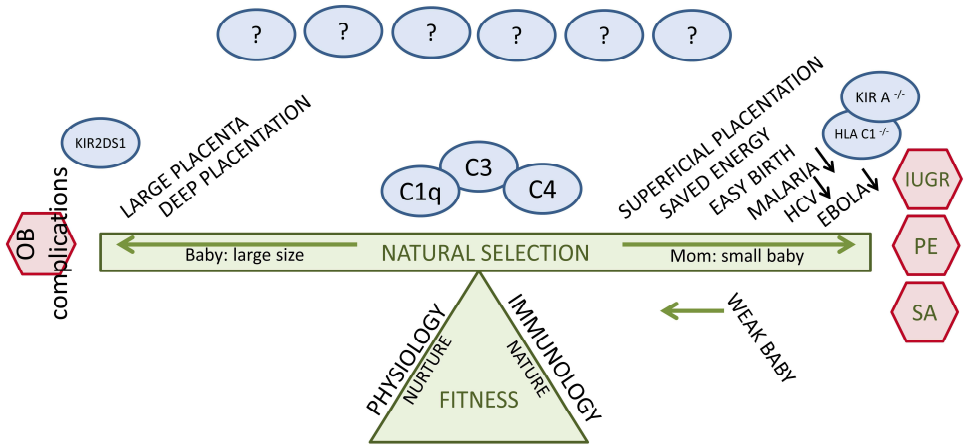

Figure 5. Suggested relationship between pregnancy complications (in red) along an evolutionary axis of balancing selection by immunogenetic and physiological characteristics. Green arrows indicated various selection pressures. KIR AA genotype is more frequently observed in survivors than fatal cases of Ebola virus (Wauquier *et al.* 2010), and they are also over-represented in patients with pre-eclampsia (Yu *et al.* 2014). HLA-C has been likewise implicated in hepatitis C virus (HCV) infections and pre-eclampsia. This study indicates, that complement components C1q (Lokki *et al.* 2014), C3 (Lokki *et al.* 2017) , and C4 (Lokki *et al.* 2014) play a part in healthy pregnancy. OB: obstetric, IUGR: intra-uterine growth restriction, PE: pre-eclampsia, SA: small for age. Blue – immunogenetic characteristics of an individual, Red – pathological endpoint, Green – evolutionary process.

The reproductive fitness of an individual is based on a combination of its immunogenetic characters and the physiological restrains of the pregnancy and childbirth. The balancing selection possibly via immunological mechanisms proposed here contains a paradoxical phenomenon of protection from severe viral infections while pre-disposing the individual to pre-eclampsia and intra-uterine growth restriction (IUGR) (Parham & Moffett 2013). There are suggestions that genes linked to tuberculosis and leishmaniosis may be novel candidate genes for pre-eclampsia susceptibility via numerous biological pathway analyses (Elliot & Crespi 2015). Similar balancing selection is observed in

the trade-off between resistance to malaria and morbidity from sickle-cell anaemia.

2.5 Maternal genetic association to pre-eclampsia

"The strongest aetiological factors in toxaemia lie in the maternal organism."
Vara, Timonen & Lokki, 1965

It has been recognised since the era pre-dating genetic research that maternal, foetal, and environmental factors all play a part in development of pre-eclampsia (Vara *et al.* 1965). Genetic association studies have addressed questions of maternal impaired immunotolerance and angiogenic imbalance for the last twenty years (Broughton Pipkin 1999). Twin studies have shown pre-eclampsia to be approximately 50% heritable (Salonen Ros *et al.* 2000).

Family linkage studies have identified several regions of maternal genetic association. Susceptibility loci in chromosome 2 have been indicated in Icelandic, Australian, New Zealander, and Finnish patients (Arngrimsson *et al.* 1999, Moses *et al.* 2000, Laivuori *et al.* 2003, Laasanen *et al.* 2003). Other candidate loci have been located in chromosomes 10, 9, 3, and 15 (van Dijk *et al.* 2005, Laivuori *et al.* 2003, Lachmeijer *et al.* 2001). Thus far, detailed studies of genes associated with the indicated chromosomal loci, have rarely resulted in confirmation of genetic association to pre-eclampsia (Kivinen *et al.* 2007, Kaartokallio *et al.* 2016, Peterson *et al.* 2009).

Genome-wide association studies have identified few susceptibility regions for pre-eclampsia. (Zhao *et al.* 2012, Johnson *et al.* 2012). Due to small sample sizes, convincing candidate genes have not been identified. Candidate gene studies have found variants that associate to pre-eclampsia in several genes in the maternal genome, but most of the candidate genes' associations have not been replicated. Among associating candidate genes are several genes that contribute to the development of the disease through numerous pathways including thrombosis, endothelial function, vasoactive proteins, oxidative stress, lipid metabolism, and immunity (Williams & Broughton Pipkin 2011).

Examples of candidate genes in pre-eclampsia include *Corin* (Atrial natriuretic peptide-converting enzyme), a gene that is abundantly expressed in the heart. *Corin* contributes to the risk of pre-eclampsia in Caucasian women with population-specific variants (Stepanian *et al.* 2014). The activin receptor genes type 1 and 2 (*ACVR1C, ACVR2A*) have been the subject of several candidate gene studies. While association to pre-eclampsia was indicated for *ACVR2A* in a Norwegian study, the results were not replicated in the Finnish population (Roten *et al.* 2009, Lokki *et al.* 2011). Two single nucleotide polymorphisms (SNPs) (rs13406336 and rs4556933) in *ACVR1C* have been considered as candidate SNPs previously, but they were not associated to pre-eclampsia in a Norwegian population (Roten *et al.* 2009).

A large meta-analysis of candidate genes in pre-eclampsia from 542 genetic association studies found associating variants in or near several candidate genes (Buurma *et al.* 2013). These included the angiotensin-converting enzyme (*ACE*), with potential to affect the regulation of blood pressure and blood volume, the lipoprotein lipase (*LPL*), which may contribute to endothelial cell dysfunction if the gene function is compromised, and cytotoxic T-lymphocyte associated protein 4 (*CTLA4*), which plays an important role in the negative regulation of T-cell proliferation and activation (Buurma *et al.* 2013). *ACE,* angiotensinogen (AGT) and angiotensin II receptor type 1 (AGTR1) were also verified as associating candidate genes in a second meta-analysis, which combined the data from 192 independent genetic association studies (Staines-Urias *et al.* 2012).

2.5.1 Finnish population genetics

Genetics of the Finnish population are unique among Europeans (Lundmark *et al.* 2008). The Finnish population structure provides an exceptional opportunity to study rare enriched variants that contribute to the risk of complex diseases (Lim *et al.* 2014). The structure of Finnish genetics is attributed to repetition of strong founder effects caused by bottleneck events (Nevanlinna 1972). Over centuries, fluctuations of internal migration created regional isolated populations in rural parts of the country, which remained surprisingly intact into the 20th century (Peltonen *et al.* 1999). Furthermore, geographic isolation has resulted in the enrichment of deleterious rare and low-frequency variants that are rare or absent in other populations (Kere 2001, Lim *et al.* 2014). This phenomenon is mirrored in what has been described as the Finnish disease heritage; a selection of 36 rare hereditary diseases (Norio 2003, Polvi *et al.* 2013). On the other hand, due to effects of negative sampling

and genetic drift, Finland has an unusually low incidence of certain genetic diseases such as cystic fibrosis, which has a ten-fold larger incidence in other Caucasian populations in comparison to the Finns (Kere *et al.* 1989).

2.6 Cellular immunity in pre-eclampsia

The predominant non-trophoblast cell population in the placenta is the Hofbauer cells (HC), which express the typical macrophage markers such as CD68 (Joerink *et al.* 2011). The HC polarise towards the alternative M2 phenotype, which are suggested to be involved in homeostasis, parasite killing, tumour promotion, tissue remodelling among other processes. While the exact role of the HC remains unknown, their numbers in HELLP syndrome are increased, likely reflecting the increased inflammation in the affected placentae (Evsen *et al.* 2013). Concordantly, a decrease in C3aR in the HC pre-eclamptic placentae may indicate the failure to clear the excess of activated complement thereby aggravating the sterile inflammation locally as immune complexes build up, which ensues in development of the disease (Lim & Lappas 2012).

In the pre-eclamptic pregnancy, the immune cell populations in the maternal tissue, the decidua, are altered. B cells and CD4+ CD29+ T-cells are increased as well as CD4+CD45RO+ memory T-cells and CD8+S6F1+ and CD8+CD28+ cytotoxic T-cells, while CD4+CD45RA+ T-cells are decreased. This results in over-production of immune complexes and autoantibodies (Matthiesen et al. 1995, Matthiesen et al. 1999, Wilczynski et al. 2003). CD4+CD25high and CD3+FoxP3+ Treg cells are decreased in comparison to normal pregnancies, which may result in loss of maternal tolerance to the foetus in pre-eclamptic pregnancies (Sanguansermsri & Pongcharoen 2008, Sasaki et al. 2007).

In pre-eclampsia, the population of natural killer t-cells (NKT) is also increased in comparison to a healthy pregnancy resulting in a shift towards Th1-type immune response (Borzychowski *et al.* 2005). The cytokine environment created by Th1-type immune response is known to be incompatible with a healthy pregnancy (Zhou *et al.* 2013, van den Heuvel *et al.* 2007).

2.7 Complement system in pre-eclampsia

The semi-allograft foeto-placental unit poses a unique challenge to the maternal immune system. Immunological mechanisms in pre-eclampsia may involve a non-classical-type incompatibility (Redman & Sargent 2010a). Maintaining clearance by complement and phagocytes, and controlling the potentially destructive effects of complement on the placenta and foetal tissues, are of central importance for a healthy pregnancy.

2.7.1 Complement activation

Complement system is an integral part of innate immunity. Complement system has several important roles. These include lysis of microbes by the membrane attack complex (MAC), opsonisation of complement activating structures by complement components C3b, C4b, and C1q, chemotaxis by C3a and especially C5a, clearance of immune complexes by C3b, C4b and CR1, initiating and supporting the adaptive immune response, and initiation, intermediation, and enhancement of immune reactions by increasing capillary permeability, inducing smooth muscle contraction, and releasing immune mediators from mast cells. Altogether the complement system comprises more than 40 different components including receptors and regulators. Its activation pathway consists of approximately 20 components that interact in a progressive cascade to instigate terminal pathway activation. Complement activation proceeds by three distinct pathways in a step-wise enzymatic cleavage of the activating components which produce chemoattractants or anaphylatoxic fragments to be released (Figure 6.).

The classical pathway (CP) is activated when the C1 complex binds to the target cell leading to activation of complement components C4, C2 and finally cleavage of complement component C3 and formation of the C5 convertase which initiates terminal pathway (TP) activation (Walport 2001a). C1q binds to apoptotic cells, immune complexes, gram-negative bacteria, some viruses, and the surface-attached C-reactive protein (CRP).

The lectin pathway (LP) of complement activation is analogous to the classical pathway. The lectin pathway is activated by mannan-binding lectin (MBL) binding to microbes with sugars such as mannose groups on the surface structures. Certain fungi including *Candida* and *Aspergillus* and a limited number of bacteria, for example certain strains of

Salmonella also bind MBL. Ficolins are the newest discovery in the complement system, they opsonise certain bacteria and yeast, and activate complement via the lectin pathway analogously to MBL (Matsushita & Fujita 2001, Endo *et al.* 2011). With the mannan binding lectin serine peptidase 1 and 2 (MASP1 and MASP2) proteins, MBL or ficolins may form an activator complex similar to C1 in the CP also leading to deposition of C4 and C2 and the formation of the C3 convertase C4b2a.

The alternative pathway (AP) is among most evolutionarily ancient immune defence mechanisms. It has the capacity to discriminate between self and non-self surfaces and particles. In AP activation, C3b is covalently bound to cell surfaces and thereafter either inactivated by soluble or surface bound complement regulators, or activated by default. Failing inhibition, AP activation will be accelerated by activation of the AP in the amplification loop where C3bBb convertases will activate more native C3 molecules to C3b to opsonise the target cell surface. During activation, Factor D (FD) will cleave C3b-bound Factor B (FB) to Ba and Bb. Together with C3b, Bb will form the C3 convertase C3bBb. AP activation will lead to the C5 cleavage and MAC formation as the final result of TP activation. MAC formation will result in cell death and tissue destruction. The surfaces lacking protective regulatory components and thereby promoting alternative pathway activation include fungi, viruses and certain bacteria (Walport 2001a).

Among the most potent surface bound regulators of the complement system is the membrane co-factor protein (MCP, CD46). The MCP is coded by *CD46,* which is located in the 1q32.2 within a genomic region called the Regulators of Complement Activation (RCA) gene cluster in the proximity of several other genes that produce complement regulators (Holers *et al.* 1985). The 43 kb *CD46* is composed of 14 exons. At least 59 amino acid changing mutations are known in the *CD46* Seven of these polymorphisms are not related to atypical haemolytic uremic syndrome (aHUS), which is a serious disease of the kidneys caused by tissue destruction resulting from un-controlled complement attack in the kidney vascular walls and on blood cells (Liszewski & Atkinson 2015, Meri 2013).

The functional region of MCP consists of four complement control protein (CCP) domains linked to a 34-amino-acid signal peptide. Thereafter, an alternatively spliced region for *O*-glycosylation, a segment of unknown function, a transmembrane domain, and one of several alternative splice variants of cytoplasmic tail follow (Liszewski *et al.* 1991).

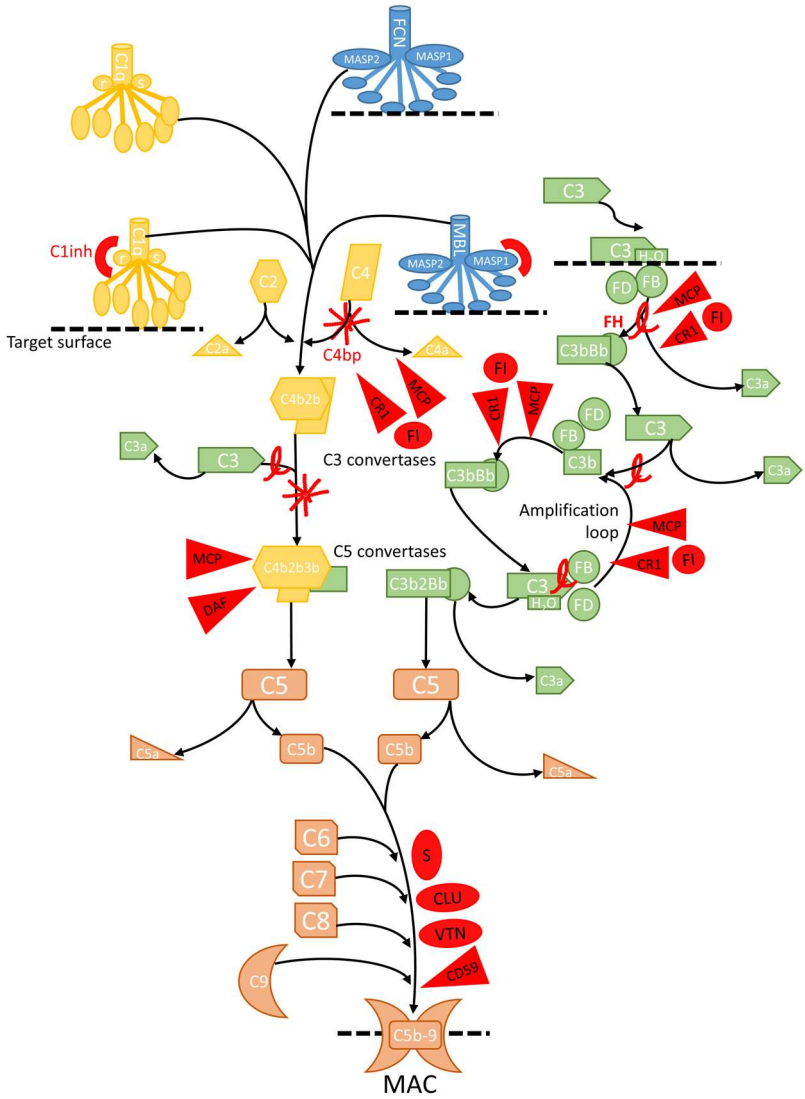

Figure 6. Complement system can be activated by alternative pathway (green), classical pathway (yellow) or lectin pathway (blue). They all lead to activation of the terminal pathway (orange) by formation of C5 convertases. The most important complement inhibitors are shown in bright red. Factor H (FH) and C4b binding protein (C4bp) are the most important soluble regulators of early pathways of complement activation. FH is particularly important in acting as a cofactor for the inactivation of C3b by factor I in the alternative pathway, while C4bp is primarily active in the classical pathway of complement activation. The triangular regulators are surface bound

inhibitors. C5a and C3a are potent anaphylatoxins with the capacity to increase vascular permeability and recruit immune cells to the site of complement activation, as well as in participating in the induction of the adaptive immune response. MAC – membrane attack complex, CLU – clusterin, VTN – vitronectin, MCP – membrane cofactor protein, DAF – decay accelerating factor, MBL – mannose-binding lectin, MASP - mannan-binding lectin serine peptidase, FCN – ficolin, C1inh – complement component 1 inhibitor, CR1 – complement receptor 1, FI – factor I, FB – factor B, FD – factor D

Other surface bound complement regulators are decay accelerating factor (DAF, CD55) and protectin (CD59). MCP facilitates the inactivation of complement components C3b and C4b by Factor I (FI), whereas DAF promotes the decay of the C3 convertase enzymes. In addition to the surface bound CD59 soluble proteins vitronectin (VTN; S-protein) and clusterin (CLU) control the terminal pathway. Important regulators of complement system are listed in Table 1 (Zipfel & Skerka 2009).

Table 1. Regulators of complement activation (modified from Zipfel & Skerka 2009).

Regulator	Location	Main target	Notes
Factor H	soluble	C3b, C3bBb	FH
FHL-1	soluble	C3b, C3bBb	FH-like protein 1
Properdin	soluble	C3bBb	Positive regulator
CR1	surface-bound	C3b, C4b	CD35
CR2	surface-bound	C3dg, C3d	CD21
CR3	surface-bound	iC3b	CD11b/CD18
CR4	surface-bound	iC3b	CD11c/CD18
CRIg	surface-bound	C3b,iC3b	VSIG4
MCP	surface-bound	C3b, C4b	CD46, membrane cofactor protein
DAF	surface-bound	C4b2a, C3bBb	CD55, decay accelerating factor
C4bp	soluble	C4b2a	
Carboxyl-peptidase N	soluble	C3a, C5a	Inactivates C3a and C5a
C1INH	soluble	C1s, C1r, MASPs	
FHR-1	soluble	C3b?, C5?	FH-related protein 1
Clusterin	soluble	C5b-9	SP40,40, ApoJ
Vitronectin	soluble	C5b-9	S-protein
Protectin	surface-bound	C5b-8, C5b-9	CD59

2.7.2 Complement in the placenta

Complement belongs to the first line of defence in the circulation and tissues. Extra-villous trophoblast cells invading into maternal tissues must be sufficiently protected from the maternal complement system (Redman & Sargent 2005, Regal *et al.* 2017). A second site in the need of protection from complement activation is on the syncytiotrophoblast, which is the foetal surface that is constantly exposed to maternal blood (Figure 7). Complement activation will result in the release of anaphylatoxins and chemotaxins with the potential to cause inflammation, vascular leakage and thrombosis. Unregulated complement activation may also result in tissue damage characterised by inflammatory lesions, and increased apoptosis on the placental villi (Ito *et al.* 2015, Rampersad *et al.* 2008).

The crucial role of complement for the healthy pregnancy is reflected in recurrent pregnancy loss, where mutations in complement regulatory genes have been reported (Mohlin *et al.* 2013). Furthermore, recurrent miscarriage and pre-eclampsia are both characterised by excessive complement activation (Denny *et al.* 2012).

The incompatibility between maternal immune system and placental cells may disrupt the balance of complement activation and regulation. This can contribute to the pathogenesis of pre-eclampsia (Girardi 2017). In discordant cases, complement attack may compromise placental cells if their protection fails. Complement activation may occur spontaneously (Meri 2016). Alternatively, antibodies or exposed tissue structures may act as the trigger. Several factors may potentially cause incompatibility between complement attack and regulation. First, excess of burden or abnormal function of the complement may limit its capacity to perform the necessary waste disposal function. Consequently, placental material cannot be properly cleared and accumulation may occur in maternal tissues such as in the lungs or blood vessels of kidneys causing inflammation and vascular damage. Secondly, antibodies, tissue degradation products, or insufficient regulation of complement activity may lead to complement over-activation with all its consequences.

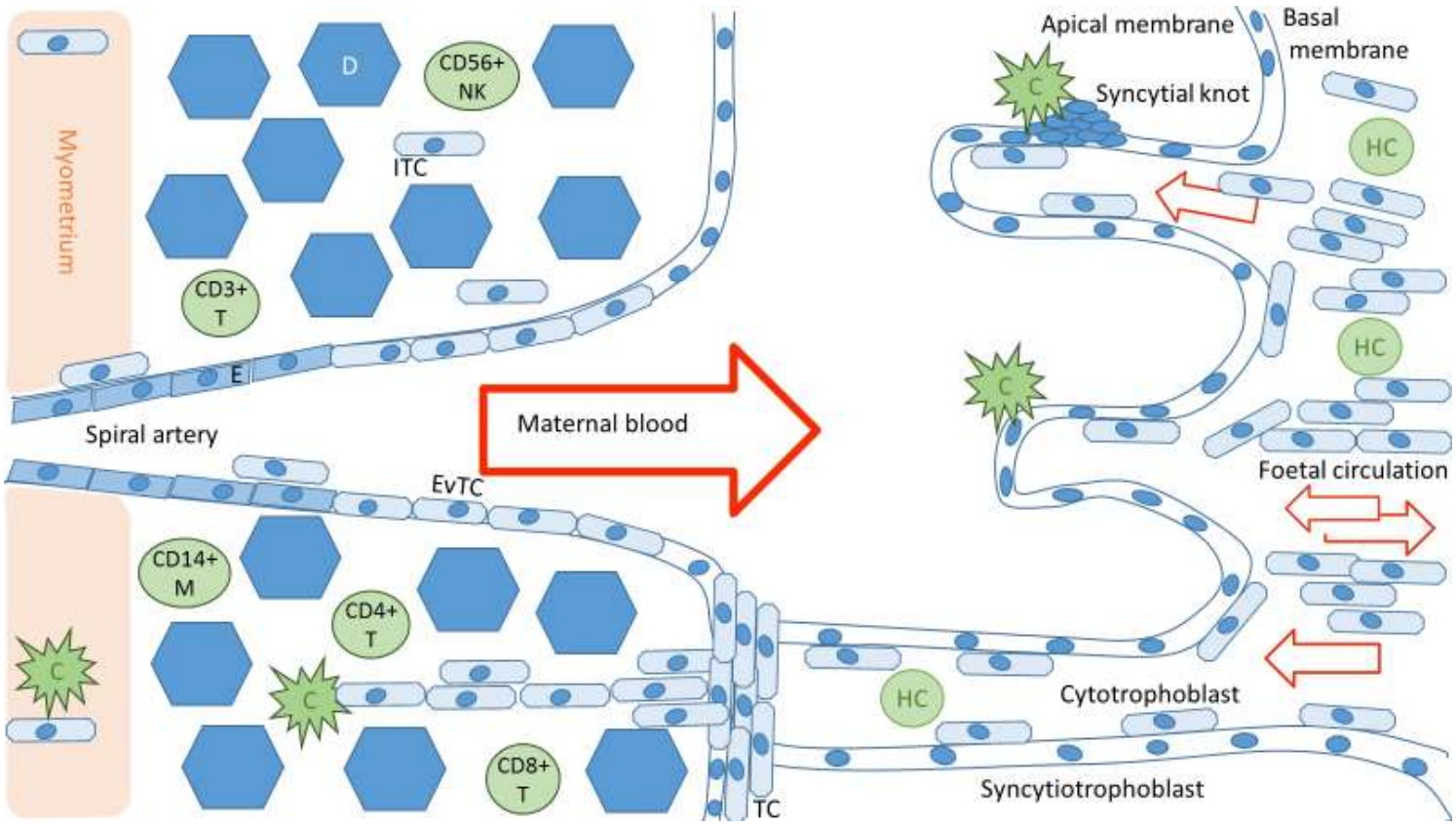
Myometrium
D
CD56+
NK
ITC
CD3+
T
E
Spiral artery
Maternal blood
EvTC
CD14+
M
CD4+
T
C
C
CD8+
T
TC
Apical membrane
Basal
membrane
C
Syncytial knot
HC
HC
C
Foetal circulation
HC
Cytotrophoblast
Syncytiotrophoblast

Figure 7. Schematic representation of the maternal-foetal interface and its immunological players (green). The placental villi are shown in the right side of the image. The syncytiotrophoblast is formed by the cytotrophoblast cells that in the early stages of the pregnancy create a double layer on the villi, in term pregnancy the cytotrophoblasts are scarce and the apical and basal membrane of the fused syncytium is the border between maternal and foetal circulation. The villi are anchored to the decidua by trophoblast columns (TC), which flatten and disappear during the course of the pregnancy fusing the anchoring villi to the decidua, which is shown on the left side of the figure. Invading trophoblast cells will encounter maternal complement system (C) in the decidua, and in the intervillous space. Successful trophoblast invasion will extend to the vascular layers of myometrium, and invade the uterine spiral arteries, where endovascular trophoblast cells (EvTC) will replace endothelial cells (E) causing remodulation and relaxation of the spiral artery to allow for non-turbulent high volume low pressure circulation into the intervillous space. Interstitial trophoblast cells (ITC) will remain in the maternal tissue creating tolerance of the foetal tissue in the maternal immune system. Hofbauer cells (HC) are the predominant immune cell population in the villi throughout placental development. The decidual immune cell population consists of macrophages (M), natural killer cells (NK), and other populations of T-cells (T). Tolerance inducing Treg and Breg cells in particular are essential for a healthy pregnancy (Ghaebi *et al.* 2017).

2.7.3 Complement disturbance in pregnancy

Complement has been studied in pre-eclampsia extensively (Gilbert *et al.* 2012, Girardi 2017, Regal *et al.* 2017). Hypertensive disorders of pregnancy with compromised placental perfusion can result from excessive activation of the complement system, or alternatively from deficiencies in complement function not allowing the adequate development and perfusion of the uteroplacental unit (Regal *et al.* 2015). The larger body of evidence points towards over activation of the complement system (Derzsy *et al.* 2010). Complement activators have been shown to be elevated in early-onset severe pre-eclampsia and late-onset severe pre-eclampsia (He *et al.* 2016). However, the results of complement studies are conflicting. Studies in support of over activation of the alternative pathway often report an excess of factor B (FB) and its activation products in the patient sera (Lynch *et al.* 2008, Hoffman *et al.* 2013), but the role of FB is not clear (Derzsy *et al.* 2010).

Disturbed complement activity can predispose to infections or to an immuno-inflammatory syndrome, which resembles systemic lupus erythematosus (SLE). SLE is associated with inadequate clearance of debris by the classical pathway (Walport 2001b). Such syndromes are likely to aggravate during pregnancy. This is possibly due to greater clearance requirement caused by shedding of material with placental origin. Furthermore, patients with SLE or antiphospholipid syndrome have an increased risk for adverse foetal outcomes and hypertensive disorders of pregnancy (Gilbert *et al.* 2012). On the other hand, inadequate regulation of the complement system can lead to catastrophic consequences such as the aHUS and other forms of thrombotic microangiopathy (TMA) (Meri 2013). In TMA, complement attacks against endogenous tissue structures such as endothelial cells and blood cells causing vascular damage and kidney failure. Pregnancy can act as a trigger of TMA syndromes.

2.8 Fms-like tyrosine kinase 1 (sFlt1) – An anti-angiogenic marker

Among the most promising lines of recent research into aetiology of pre-eclampsia is the investigation of the role of angiogenesis in the pathophysiology of pre-eclampsia (Agarwal & Karumanchi 2011). Two of

the best documented increases in anti-angiogenic factors are seen in soluble endoglin (sEng), a transforming growth factor-β1 and -β3 receptor, and soluble fms-like tyrosine kinase 1 (sFlt1) a splice variant of vascular endothelial growth factor receptor 1 (VEGFR1) (Levine *et al.* 2006). SFlt1 is a known anti-angiogenic marker of pre-eclampsia that is predominately expressed by the trophoblast cells. VEGFR1 is coded by fms related tyrosine kinase 1 gene (*FLT1*). Multiple isoforms of VEGFR1 exist, and the soluble forms have been implicated in pre-eclampsia. The extracellular parts of the protein are encoded by the first 13 of 30 exons. Intracellular parts coded by the latter half of the gene are not included in the soluble VEGFR1. The protein in its entirety consists of seven immunoglobulin (Ig)-like domains in an extracellular ligand-binding region, a transmembrane segment and a cytoplasmic region containing a tyrosine kinase (TK) domain (Shibuya 2001). Endothelial tissue throughout the body produces VEGFR1.

An excess of VEGFR1 of placental origin has been recorded in pre-eclampsia (Maynard *et al.* 2003). While prediction of pre-eclampsia has proven difficult, the best results have been obtained by observing the ratio of sFlt1 to placental growth factor (PlGF) (Zeisler *et al.* 2016). SFlt1 levels typically correlate with the severity of pre-eclampsia (Levine *et al.* 2004), but conflicting views persist in relation to the normalisation of sFlt1 levels in pre-eclampsia. Previously, sFlt1 levels were considered to normalise after the birth of the placenta (Levine *et al.* 2004, Levine & Karumanchi 2005). However, recently it was shown that in some patients sFlt1 levels may remain elevated in women with a history of pre-eclampsia even after healthy endothelium is restored (Tuzcu *et al.* 2015).

2.8.1 Role of VEGFR1- mediated angiogenesis in placentation

VEGFR1 is essential for survival through its capacity to negatively regulate the levels of VEGF. Internalisation and signalling of functional VEGF receptors enhances angiogenic growth of blood vessels (Boulanger 2016). VEGFR1 also binds PlGF thereby limiting its availability and proliferation of the placental tissue. VEGFR1 has been shown to be expressed at the site of implantation, where blocking the VEGFR1 had immunomodulary effects i.e. reducing the recruitment of macrophages to the uterus, although pregnancy itself was not compromised by the anti-VEGFR1 treatment in a murine model (Douglas *et al.* 2014). Furthermore, by continuous blocking of VEGFR1, angiogenesis of the implantation site was reduced by half (-48% vascular density) (Douglas *et al.* 2014).

2.8.2 sFlt1, PE, and comorbidity

Pre-eclampsia is associated with increased risk of non-communicable diseases in later life. Women with a history of pre-eclampsia have at least double the risk of a future cardiovascular CVD in comparison to healthy controls, and the risk increases with severity of the disease (Ray *et al.* 2005, Bellamy *et al.* 2007). The heightened risk of coronary artery calcification is detectable three decades after the index pregnancy (White *et al.* 2016). Moreover, an increased risk for stroke (Bushnell *et al.* 2014) and type 2 diabetes has been observed after a pre-eclamptic pregnancy (Weissgerber & Mudd 2015).

For healthy circulation to persist, sustaining angiogenesis is paramount. Cardiovascular morbidity is observed accordingly in association with anti-angiogenic treatment (Abdel-Qadir *et al.* 2017). The driver of the cardiac dysfunction in pre-eclampsia is likely mediated by anti-angiogenic factors (Shahul *et al.* 2016). Pre-eclampsia also increases the risk of peripartum cardiomyopathy, and it has been suggested that sFlt1 may be toxic to the heart. Increased levels of sFlt1/sVEGFR1 are associated with peripartum cardiomyopathy with correlating levels of sFlt1 and symptom severity (Damp *et al.* 2016). Higher levels of sFlt1 are also recorded in heart failure after myocardial infarction in absence of pregnancy (Onoue *et al.* 2009). In heart failure concurrent with pregnancy, extreme sFlt1 levels are also observed (Wu *et al.* 2017). During the time period of 1-10 years after pre-eclampsia, 3.6-fold increase in risk of heart failure is associated with pre-eclampsia (Wu *et al.* 2017). Severe pre-eclampsia bears the largest risk for cardiomyopathy in later life in comparison to GH (second largest risk) or moderate pre-eclampsia (smallest risk) (Behrens *et al.* 2016).

2.8.3 sFlt1 - evolutionary context

While pre-eclampsia is a primate-specific disease, sFlt1 is conserved in vertebrates across avian and mammalian classes. The major contributor to sFlt1 load in human pregnancy is the recently evolved isoform sFlt1-e15. Overexpression of the primate specific isoform sFlt1-e15a is also associated with pre-eclampsia, suggesting that this novel isoform harbours thus far unexplained fitness advantages (Thomas *et al.* 2009). Assuming that sFlt1 is pathogenic, it is thereby possible that in non-primate mammals' pregnancy-associated pathologies, the rise in sFlt1 does not exist. On the other hand, it is also possible that the sFlt1 in

humans has evolved specific functions, patterns of expression, or regulatory mechanisms that are essential for development of pre-eclampsia (Brown *et al.* 2013).

sFlt1 has been implicated to have an anti-inflammatory function (Yano *et al.* 2006). It is also expressed on inflammatory cells (Luttun *et al.* 2002). In areas of Africa where, *Plasmodium falciparum* is endemic, first pregnancies share a particular risk of not only pre-eclampsia but also of placental malaria (Muehlenbachs *et al.* 2008). Interestingly, it has been shown, that in placental malaria the foetal tissue will express an excess of sFlt1 apparently in an attempt to regulate the maternal inflammatory response thereby reducing the rate of spontaneous abortions (Muehlenbachs *et al.* 2006). Consequently, positive selection on a genetic variant with capacity to resist placental malaria may have influenced allele frequencies within the general population enough to introduce a novel risk to pre-eclampsia (Brown *et al.* 2013). Further evidence of the immunological interactions of VEGFR1 come from a murine model, where increase in complement activation resulted in increased levels of VEGRF1 (Girardi *et al.* 2006). It was confirmed that monocytes can be stimulated to express an excess of VEGFR1 when exposed to complement activation products C3a and C5a *in vitro* (Girardi *et al.* 2006).

2.9 Animal studies in pre-eclampsia

In studies of pregnancy complications, most invasive research techniques and many clinical trials are impossible to carry out safely during pregnancy. This makes the concept of animal model studies enticing. Indeed, rats and mice have been used to reproduce particular symptoms of the syndrome-like pre-eclampsia using numerous methods. For example: an injection of an adenovirus carrying sFlt1 to pregnant mice recapitulates hypertension and foetal growth restriction (Lu *et al.* 2007). This model has been used to study the VEGF and PlGF pathways in pre-eclampsia pathology, the connection of sFlt1 increase to the well-documented increase in CVD risk in later life and even the beneficial effect of CO in pre-eclampsia (Mateus *et al.* 2011, Venditti *et al.* 2014, Pruthi *et al.* 2015). The role of sFlt1 in pre-eclampsia pathogenesis was originally confirmed in Sprague-Dawley rats using a similar protocol (Maynard *et al.* 2003). Recently, a second mouse model of late-onset pre-eclampsia has been developed with the overexpression of human sFlt1-e15a (Szalai *et al.* 2015).

Another area of recent advances in pre-eclampsia studies via animal models is the molecular mechanisms to mitigate vascular pathologies in the pre-eclamptic placenta using reduced uterine perfusion pressure (RUPP) rats or chemically created pre-eclampsia-like state of decreased vasodilation in BALB/c mice (Morton *et al.* 2015, Motta *et al.* 2015).

Some of the most compelling evidence of the link connecting innate immunological mechanisms to onset of pre-eclampsia comes from animal studies. Already in 2000, regulation of C3 was shown to be crucial for a successful pregnancy in a revered experiment using Crry-/- and C3-/- mice (Xu *et al.* 2000). One of the most complete models for pre-eclampsia is reproduced in pregnant C1q-deficient (C1q -/- -strain) mice (Singh *et al.* 2011). Activation of the alternative pathway of the complement system has been shown to be crucial in development of hypertension in the RUPP rats (Lillegard *et al.* 2013). Furthermore, blocking of activation of the complement system in known areas of complement activation will prevent maternal features of pre-eclampsia in DBA/2-mated CBA/J mice (Qing *et al.* 2011).

A serum-based pregnancy-specific C57BL/6 IL-10−/− mouse model that closely mirrors pre-eclampsia is created by a single administration of human pre-eclampsia serum (Kalkunte *et al.* 2010). These mice mimic many of the symptoms of pre-eclampsia including placental hypoxic injury and increased sFlt1 and endoglin levels thus confirming the role of immunological pathways in aetiology of the disease (Kalkunte *et al.* 2010).

It is noteworthy, however, that development of pre-eclampsia is intrinsically linked to insufficiency of deep placentation unique to the higher primates (Burton *et al.* 2015). For example, in the RUPP model, compromised blood flow mimicking conditions during pre-eclampsia is created by mechanically blocking blood flow via the uterine arteries using clips fitted inside pregnant rats in a surgical procedure. While similar pregnancy complications to mild pre-eclampsia and GH are consequently recreated, the RUPP model does not reflect the systemic endothelial dysfunction typical of severe pre-eclampsia or its rare complications (Balta *et al.* 2011).

3. AIMS AND OBJECTIVES

In this study, I assessed the role of complement system and candidate genes in pre-eclampsia.

The specific aims were:

1) Assess the genetic role of C3 in severe pre-eclampsia.
2) Assess the genetic role of MCP in severe pre-eclampsia.
3) Describe complement components' expression and deposition on the placenta tissue of normal pregnancy, late-onset pre-eclampsia and early-onset pre-eclampsia.
4) Evaluate the role of genetic polymorphisms in candidate genes in the spectrum of pre-eclampsia and their comorbidity.

4. MATERIALS AND METHODS

This study was conducted at the Departments of Medical and Clinical Genetics and Bacteriology and Immunology, and at Immunobiology Research Program, and at Institute for Molecular Medicine Finland in the University of Helsinki. Laboratory studies for Study IV were conducted in collaboration with Institute for Molecular Medicine Finland and School of Medicine, Washington University in St. Louis, MO, USA. Statistical analysis for Study IV were conducted in collaboration between Institute for Molecular Medicine Finland and Broad Institute, MA, USA.

This study consists of four case-control studies of pre-eclampsia in the Finnish population. Controls were defined as non-pre-eclamptic singleton pregnancies. Pre-eclampsia presents with variable symptoms at different time points during the second half of the pregnancy. Hypertension and proteinuria were compulsory symptoms of pre-eclampsia according to the definitions available during the recruitment years of the included studies. The convulsive state, eclampsia, is usually but not always preceded by pre-eclamptic symptoms. Neurodamage, kidney damage, foetal demise, and death are severe complications of pre-eclampsia. In some cases, typically associated with HELLP syndrome, liver failure is seen. HELLP is often preceded by pre-eclampsia diagnosis, but may also occur without hypertension and proteinuria.

4.1 Definitions of pre-eclampsia sub-phenotypes

In this study, we used guidelines from the FINNPEC study to determine the subphenotypes of patients with pre-eclampsia (Jääskeläinen *et al.* 2016). Early vs. late onset pre-eclampsia may be defined by either the time of diagnosis or delivery. In study I, we used the time point of diagnosis earlier or later than 34+0 pregnancy weeks to define early and late onset pre-eclampsia. The diagnostic criteria for patients in each study are outlined in table 2.

Table 2. The diagnostic criteria for defining groups of pre-eclampsia patients and controls in each study.

	Systolic blood pressure, mmHg	Diastolic blood pressure, mmHg	Proteinuria, g/L or per 24h sample (repeated dipstick reading)	Diagnosis week	Study where used
Early-onset pre-eclampsia	≥ 140	≥ 90	≥ 0.3 (≥ 1+)	< 34+0	I
Late-onset pre-eclampsia	≥ 140	≥ 90	≥ 0.3 (≥ 1+)	> 34+0	I
Severe pre-eclampsia	≥ 160	≥ 110	≥ 5	na	II and III
Non-severe pre-eclampsia	≥ 140	≥ 90	≥ 0.3 (≥ 1+)	na	I and IV
Controls	< 140	< 90	none	na	I - IV

4.2 Study subjects

4.2.1 FINNPEC

This study is part of larger pre-eclampsia research consortium, Finnish Genetics of Pre-eclampsia Consortium (FINNPEC). In three of the studies presented here, we have used exclusively samples from the FINNPEC cohort (Jääskeläinen *et al.* 2016). In Study III, control individuals from the FINNPEC cohort were combined with patients from the Helsinki study cohort, because in the beginning of the study the review of the FINNPEC hospital records of the patient group were still ongoing. In FINNPEC, DNA samples and clinical data have been collected from all university hospitals in Finland (Helsinki, Turku, Tampere, Kuopio and Oulu) beginning in 2008. First and third trimester serum and placental samples after delivery, including 9-site biopsies for transcriptome studies, have been collected from a subset. The inclusion criteria for pre-eclampsia are in accordance to standard criteria based on hypertension and new onset proteinuria occurring after 20 weeks of gestation (ACOG Committee on Obstetric Practice 2002). Hypertension is defined as systolic blood pressure of 140 mmHg or more, and/or a diastolic blood pressure of 90 mmHg or more after 20 weeks of gestation. Proteinuria is defined as the urinary excretion of ≥0.3g protein in a 24h specimen, or 0.3g/l, or in the absence of concurrent quantitative measurement, at least a '2+' or more, or two '1+' proteinuria dipstick readings with no evidence of the urinary tract infection. Pre-eclampsia is considered severe if blood pressure is ≥160/110mmHg, or proteinuria exceeds 5g/24h, or subjective symptoms such as cerebral or visual disturbances or abdominal pain appear. Intrauterine growth restriction/placental insufficiency is defined as birth weight below -2SD and/or umbilical artery resistance ≥+2SD according to gestational age specific standards (IUGR) (Acharya *et al.* 2005) without known aetiology unrelated to the aims of the present project (e.g. congenital malformation syndromes and chromosomal defects). The women with multiple pregnancy and maternal age less than 18 years are excluded. All diagnoses were confirmed by an independent double jury consisting of a research nurse i.e. midwife and a medical doctor i.e. resident or specialist in gynaecology and obstetrics using the customised diagnostic chart Figure 8.

finnpec

identification code	

DIAGNOSIS CHART – JURY

≥ 18 years old		Single pregnancy	
Pre-eclampsia		Multiple pregnancy	

Does not match patient group criteria	

When did BP first develop: date_____ h _____ recruited patient__
When did proteinuria first develop: date_____ h _____ rerospective patient___
BP highest measurement: _____/_____ date _____ control___
Delivery: date _____ h _____

		no = 0	not severe = 1	severe = 2
Pre-eclampsia BP ≥ 140/90 and proteinuria. Severe (one or more): BP ≥160 and/or /110, proteinuria ≥ 5g/d, subjective symptoms -> preferably findings	A			
		no = 0	yes = 1	
Early pre-eclampsia < h34+0	B			
Chronic hypertension	C			
Gestational hypertension BP elevated ≥ 140/90 in absence of proteinuria	D			
Gestational DM (diet)	E			
Gestational DM (insulin)	F			
HELLP syndrome (hemolysis, asat and alat elevated, thrombocytopenia)	G			
Eclampsia	H			
SGA (- 2 sd)	I			
Normal pregnancy	J			
Foetus mortus	K			
Placental insufficiency (PI > + 2 SD or RI > +2 SD)	L			
Proteinuria during pregnancy in absence of hypertension	M			
DM type I before pregnancy	N			
DM type II before pregnancy	O			
Other pregnancy complication	P			
		+ = yes - = no	if yes, what	
Kidney disease				
Immunological disease				
Final FINNPEC jury code:				
Jury signature: Date:				
Diagnoses:				

Data in access	
Antenatal chart scanned	
Quick database complet.	
Full database completed	

Figure 8. The diagnostic chart used by midwife/resident double jury in the FINNPEC procedure.

4.2.2 The Finnish population-based pre-eclampsia cohort

The Finnish population-based pre-eclampsia cohort consists of 248 pre-eclamptic women and 679 controls identified and recruited from 100,000 consecutive pregnant women from the National Hospital Discharge Register. While the pre-eclampsia group followed diagnostic criteria described in Table 2., the control group consisted of uncomplicated pregnancies and women with non-pre-eclamptic pregnancy complications at comparable proportion to the general population. The cohort is described in detail elsewhere (Hiltunen *et al.* 2009).

4.2.3 Southern Finland pre-eclampsia study

The Southern Finland pre-eclampsia study cohort consists of 113 women who had had pre-eclamspia in at least one previous pregnancy and 103 controls with at least one normotensive pregnancy. The cohort consists of previously healthy women of Finnish origin who lived in Southern Finland without a history of renal or autoimmune history. The cohort has been described in detail previously (Laivuori *et al.* 2000).

4.2.4 FINRISK

FINRISK is a large Finnish population survey for the purpose of study of risk factors on chronic, noncommunicable diseases. The study was initiated in 1972, but for this study, we used the comprehensive National Hospital Discharge Register covering years 1992 to 2007 to identify pre-eclamptic cases and control pregnancies within the FINRISK study. The FINRISK study is described in detail online [a].

4.2.5 Patient selection in Studies I-IV

For Study I, placental samples were chosen preferentially from FINNPEC patients with severe and early-onset pre-eclampsia, with previous diagnosis of an immunological disorder as an exclusion criterion. We divided the pre-eclampsia patients in early- and late-onset groups according to the weeks of gestation at diagnosis (early-onset < 34 weeks of gestation, late-onset ≥ 34 weeks of gestation). Two patients had HELLP syndrome. Chronic hypertension (an elevated blood pressure that predated the pregnancy or detected before mid-pregnancy) was observed in one person in every study group. One patient of the late-onset pre-eclampsia group had celiac disease and one patient of the early-onset pre-eclampsia group had thrombophilia, (Factor II mutation).

In Study II, 95 severe pre-eclamptic women with severe proteinuria (minimum 5 g/L) and 190 controls were selected from the FINNPEC cohort. Patients and controls had similar BMI, but control women were slightly older (29 vs. 30.8 years p=0.003) and less often primiparous (100% vs. 45%) (p<0.001).

In Study III, a combination of samples from the three study cohorts, the FINNPEC cohort, the Finnish population-based pre-eclampsia cohort and Southern Finland pre-eclampsia study, were used.

In the SNP genotyping two datasets (the Finnish population-based pre-eclampsia cohort and Southern Finland pre-eclampsia study) were used with a total of 259 women with pre-eclampsia and 426 control women. 32 patients from the Southern Finland pre-eclampsia study were selected for the Sanger sequencing and replication was done with the severe pre-eclampsia patients (N=95) from Study II, results were compared against controls from the FINNPEC cohort (N=95). In sequenom genotyping, we used 960 severe pre-eclamptic women and 705 controls from the FINNPEC cohort to increase the sample size in 14 SNPs within the gene.

In Study IV, geographically matched patients (N=500) and controls (N=190) from the FINNPEC cohort were studied. The patients were non-obese (BMI < 30 kg/m2) without known immunological diagnoses. Genotypes from 122 women with a history of pre-eclampsia from the national FINRISK cohort were added as cases. Their diagnoses were verified using The International Classification of Diseases (ICD) codes from the Care Register for Health Care. Furthermore 1905 parous women without a history of pre-eclampsia were included as controls. For the final association analyses, we combined genotypes from sequenced FINNPEC and FINRISK cohorts, which combined totalled 615 cases and 2094 controls.

4.3 Ethical considerations

All subjects provided a written informed consent in accordance with the Declaration of Helsinki. All study subjects were at least 18 years of age and had sufficient Finnish or Swedish skills to understand the sampling protocol and study questionnaire. Study protocols were approved by the local Ethical Committees: for the FINNPEC study, ethical approval has been obtained from the Coordinating Ethics Committee, Hospital District of Helsinki and Uusimaa, for the Finnish population-based pre-eclampsia

cohort was approved by the ethics committee of the Finnish Red Cross Blood Service and by the Ministry of Social Affairs and Health and for Southern Finland pre-eclampsia study was approved by the Ethics Committee of the Department of Obstetrics and Gynaecology at Helsinki University Central Hospital. Analyses were done using coded data sheets to ensure anonymity of the patients and traceability to primary database if necessary. The FINRISK ethical approvals are available online [b].

4.4 Laboratory methods

4.4.1 Immunohistochemistry (Study I)

Placental tissue studies were done using unfixed 5 µm cryo sections from placenta tissue samples removed from the middle of the organ. Placental samples of approximately 1 cm square cylinders were frozen within two hours after the delivery in liquid nitrogen using nested isopropanol mediated protocol to minimize complement shedding during sample handling. After initial 20-minute freezing at the nested isopropanol container, cryotubes were stored at -80 °C until tissue sectioning using a cooled cryotome. Haematoxylin and eosin (HE) stain was done for all placental sections. All stainings had a mock negative control along the staining (I antibody omitted) and standard immunofluorescent staining protocol was followed using a darkened humidity chamber at RT for incubations. The classical, alternative and terminal complement pathways were evaluated using the antibodies listed in Table 3.

4.4.2 DNA extraction (Studies I-IV)

DNA was mostly extracted from 10 ml EDTA whole blood stored at-20C° (after initial freezing period at-80C° aimed to prevent formation of icicles resulting in molecular degradation) using Chemagic Magnetic Separation Module I (Chemagen, PerkinElmer, Baesweiler, Germany) automatic DNA extraction protocol as provided by the manufacturer.

DNA for some samples was extracted using standard phenol-chloroform extraction protocol from 10 ml EDTA whole blood samples. Extracted DNA was used at final concentrations of 20 ng/µl and 30 ng/µl.

Table 3. Primary antibody dilutions and types according to role in the complement system.

Role	Name	Dilution / Source*	Type
Classical pathway			
regulator	Cabp	1:200 / The Binding Site	Sheep pAb
component	C1q	1:1000 / DAKO	Rabbit pAb
component	C4c	1:400 / DAKO	Rabbit pAb
activator	CRP	1 µg/ml / Fitzgerald	Goat pAb
Alternative pathway			
regulator	Factor H	1:400 / Calbiochem	Goat pAb
component	C3c	1:1000 / DAKO	Rabbit pAb
component	C3d	1:1000 / DAKO	Rabbit pAb
Classical and alternative pathways			
regulator	MCP (CD46)	1 µg/ml / IBGRL	Mouse mAb
regulator	Bric 230 (CD55)	1 µg/ml / IBGRL	Mouse mAb
regulator	CR1	1 µg/ml / AbD Serotec	Mouse mAb
Terminal pathway			
regulator	Bric 229 (CD59)	1 µg/ml / IBGRL	Mouse mAb
component	C9	1:400 / Quidel	Goat pAb
Pre-eclampsia			
indicator	s-Endoglin	2 µg/ml / Santa Cruz	Goat pAb

**DAKO, Glostrup, Denmark; The Binding Site, Birmingham, UK; Fitzgerald Industries International, North Acton, MA, USA; Calbiochem, Merck KGaA, Darmstadt, Germany; Quidel Corporation, San Diego, CA, USA; IBGRL The International Blood Group Reference Laboratory, Bristol, UK; AbD Serotec, Oxford, UK; Santa Cruz Biotechnology, Inc., Dallas, TX, USA*.

pAb, polyclonal antibody; mAb, monoclonal antibody.

CP, classical pathway; AP, alternative pathway, TP, terminal pathway; PE, pre-eclampsia.

4.4.3 SNP genotyping (Studies II and IV)

An Illumina sequenom custom made SNP array was designed by Dr. Ville Holmberg (University of Helsinki). The SNP selection for the array was based on known SNPs in genes coding for components of the complement system that have been cited in literature to have pathological associations. In quality control analysis, all SNPs passed the missingness test with at least 80% genotyping success. Twenty-one SNPs failed the frequency test Minor allele frequency (MAF) < 0.05 and the remaining 72 SNPs entered the association analyses. A second Sequenom iPLEX genotyping was done to increase the sample size of 14 SNPs observed in C3 Sanger sequencing. On both occasions, the assay design and the genotyping were performed with Sequenom MassArray system at the FIMM Technology Centre, University of Helsinki. The Technology Centre performed routine quality control steps to ensure high quality of the genotyping.

4.4.4 Sanger sequencing (Studies II-III)

Following the manufacturer's protocol, PCR was carried out using Mytaq (Bioline, London, UK) or AmpliTaq Gold (Thermo Fisher Scientific, Waltham, MA USA) polymerase enzymes with manufacturers' buffer. The primers used in Sanger sequencing are listed in Table 4. Purification of PCR products was done with ExoSAP-IT (GE Healthcare Life Science, UK) shrimp phosphatase alkaline product. The purified DNA fragments were sequenced according to manufacturer's protocol using Big Dye Terminator v3.1 Cycle enzyme and buffer (Applied Biosystems, Carlsbad, CA, USA). The final sequencing reaction product was purified and analysed at FIMM sequencing core facility by Performa DTR v3 filterplates (Edge BioSystems, Gaithersburg, MD, USA) and ABI3730xl capillary electrophoresis sequencer (Applied Biosystems, Carlsbad, CA, USA).

Table 4. The primers used for Sanger sequencing in this study. The capitalised font refers to forward strand and non-capitalised font to reverse strand sequences. *CD46* – membrane cofactor protein (MCP), *C3* – complement component 3

Gene	Region	Left primer	Right primer	Amplicon Size
CD46	promoter	CCGAATTCCCGGAAACTATT	CCGGAGAAGGAGTACAGCAG	561
CD46	exon 1	TCGGTTTCTCTGCTTTCCTC	AGAGAACCCTGTCCCCAAAC	265
CD46	exons 2-3	TTCCCAAACAAACCAAAAGC	CCCTTATTTCCTCTAAGGAGCA	877
CD46	exon 4	GTGGAAAGGCACAGCAGATT	GGGTGTAAAGGAGGCAAAAA	398
CD46	exon 5	TTGACAAATTTATTGAAGACACAGAA	CAGGAGGAGGAAGCACATACA	392
CD46	exon 6	TTGCATTCCATTCCTTGTCTC	TCTAAAATGAACAGCAACAACAA	348
CD46	exons 7-8	AACTCCCAAGTGGTTGATCTTC	CAAATGTCCTCCCTCCTTTC	385
CD46	exon 9	TTGATAAGGCCCTGGTGAAT	CACGCTGTGCACACATACC	180
CD46	exon 10	AAGGGATTTTCTACAAAGGTGAA	TGTTTGGGCACCTCATAAAA	240
CD46	exons 11-12	TCTGGAGATCCATGTGTTCAA	ACTGAAGCTGCACAAAAGCA	730
CD46	exon 13	TGCTACTCGTTTCTTTTTGGTTT	AGCAAAGGGAACAGGAATGT	299
CD46	exon 14.1	CCAGGTTGGTGGCTCATTAC	TTTTTATGCACAAGAGCCAAA	679
CD46	exon 14.2	TCTTTGTAAAGAAAGTGGCTTGAA	GCCAAGGCAATGTAAATGGA	658
CD46	exon 14.3	AGTCTTGTTGTTTTCCCAAAGA	GAATTCCTGTTTGTTCTCCTCAA	694
CD46	exon 14.4	TTGGGCCAAAGAAACATTG	GATGGGCCCAATTAGAAACA	700
C3	Promoter	GGGAGCCTCCTTGGAAAATA	AGCCCTGTTTGTGGGTAGAG	852
C3	Exon1	CTGCTCACTCCTCCCCATC	AAATGTCTGCTTCCACCCC	200
C3	Exon2	GAGGACTGGCGTCTCACATC	ggaggggctcaggaggag	328

C3	Exon3-4	CAAGATCCGGAAGCTGGAC	TTGCCTCTCCTAAGCCTGTG	446
C3	Exon5-7	AGCTGAGAGGCTAAGCCCAG	GTCTTCACCTGGTCCCTCAC	579
C3	Exon8-9	GGAGATCCCATTCTCCAGG	CTTCTGACCTGGTCTCCCC	450
C3	Exon10-11	GGAGGTCTAATCCTGAGGGG	GAACCCCTGTACCGTCTTCC	479
C3	Exon12-13	caccaattcccaggtctcag	agacagttgagagacagagaggg	807
C3	Exon14	CATCCCAGGCACTCCTCTC	CTCCAGTCCCACCCACCTC	299
C3	Exon15-16	GTGGGGTCATTTGGGAAGAG	TCCCCTCCTCCCTCTCTG	652
C3	Exon17	GAAGTCCTCCCTGGGGTC	TCCCTCCTCAGACAGGAGTC	357
C3	Exon18-19	ccaactcctggcctcaag	ATGACACTCAGACACCCTGG	630
C3	Exon20-21	AAGAGCTGAGACCCAGGAGC	GAAGACCAGGAGCCCTCTC	583
C3	Exon22-23	TGCTGACCATCTGTGTGTCTG	ATGAGATGGAATTTGGCTCC	420
C3	Exon24	AACCCTTTTCACGCCACC	GGGATCTTAGGGGAGGGATG	344
C3	Exon25-26	CTGTCCCCTCTCTGCACC	CTCTCGTGTTCATCCTGCG	726
C3	Exon27	GATGACTGCCATGTGTGGAC	CAAGGTGGCTGTGCTCTG	222
C3	Exon28-29	TCCTTACTAACGTGACAGCAATG	CTAGGAGGCCAGTGGGAAG	906
C3	Exon30-33	GATGTCCCAGCTCTGATTTG	ACTTGGAAAGTACTGAATATCATGG	954
C3	Exon34-35	TCCTTGTCCAGGAACAGACC	CAGCCAGATAGAGGTCAGGG	423
C3	Exon36	CCCAAGACAATGCTGGACTC	CCCCACAATTCATATATACCTGG	246
C3	Exon37-38	TGGTCTTTGGAGGGAGGC	CAACCACACCTACCACCCAC	504
C3	Exon39-41	GTGCCCCTCATGGTCAAC	GGCAAAGAACTCCAGACACG	697

4.4.5 Microsatellite analysis (Study III)

GF100472 is a $(CA)_n$ repeat polymorphism in the promoter area of *C3* gene. Size of the $(CA)_n$ repeat was determined by fragment analysis. The are of CA-repeats was amplified by PCR using forward primer with FAM –label and non-labelled reverse primer in PCR conditions of an initial denaturation at 95°C for 10 minutes, followed by 32 amplification cycles of 95°C for 30 seconds, 67°C for 30 seconds and 72°C for 50 seconds and final extension at 72°C for 7 minutes. The primer used were FAM-ATGGGAGGAAGACCACCTTT/forward and CCCCTCACTTACCCTTGTCA/reverse, which produced fragments of 245-259 nucleotides. FIMM Sequencing Laboratory determined the sizes of the amplified fractions with an automated capillary sequencer ABI3730xl DNA Analyzer (Applied Biosystems). GeneScan 500 LIZ Size Standard (Applied Biosystems) was applied to size the fragment data. The number of CA repeats was verified with Gene Mapper v4.0 software (Applied Biosystems).

4.4.6 C4 gene quantification (Study I)

The normal gene number for *C4A* and *C4b* is considered to be two copies of each, although it is not uncommon to find 0-4 copies of either gene in a healthy individual. In an attempt to correlate the C4 deposition observed in the placentae with the functional *C4* genes, *C4A* and *C4B* gene copy numbers and a silencing *C4A* mutation were analysed using a previously published protocol (Paakkanen *et al.* 2012). In essence, a real-time quantitative polymerase chain reaction with a specified concentration range approach was used to obtain numbers of *C4* and to detect deficiencies due to CTins, which renders the affected *C4A* non-functional. An individual with 0-1 copies of *C4A* or *C4B* or with the insertion polymorphism within the *C4A* is considered deficient in C4.

4.4.7 Targeted exome sequencing (Study IV)

Targeted exome sequencing was performed at Washington University School of Medicine, St. Louis, MO, USA. Genomic DNA was prepared into libraries using in-house protocols and enzymes from Enzymatics (Beverly, MA) were used (Triebwasser 2015). In sequence capture by hybridisation, manufacturer's recommendations were modified to add longer blocking oligos containing 7bp inosine segment to enable promiscuous pairing with different index sequences (Roche SeqCap

Hybridization and Wash Kit No. 05634261001). Captured DNA was washed and eluted after hybridisation following the manufacturer's instructions. The targeted genes are listed in Table 5.

Table 5. List of genes and loci that were studied by targeted exomic sequencing.

Candidate genes					Candidate loci	
					Gene	SNP
ACE	*AGTR1*	*ERAP2*	*JAG1*	*ROCK2*	*AGT*	rs699
ACVR1	*AGTR2*	*ESRRG*	*KDR*	*SOD1*	*AGT*	rs4762
ACVR1B	*ANGPT1*	*F13A1*	*KIAA1239*	*SOD2*	*AGTR1*	rs5186
ACVR1C	*ANGPT2*	*FLT1*	*KIAA1462*	*STOX1*	*APOE*	rs429358
ACVR2A	*ANGPT3*	*FLT4*	*LCT*	*STOX2*	*CTLA4*	rs231775
ACVR2B	*ANGPT4*	*FN1*	*LIPA*	*SWAP70*	*ESRRG*	rs17686866
ACVRL1	*ANTXR1*	*GPR98*	*LMCD1*	*TGFB1*	*IFLTD1*	rs10743565
ADAM10	*Cdkn1c*	*HEY1*	*LPL*	*TGFB2*	*IL10*	rs1800896
ADAM12	*COMMD7*	*HEY2*	*LRRFIP1*	*TGFB3*	*KIAA1239*	rs1426409
ADAM15	*COMT*	*IFLTD1*	*MAGI1*	*TGFBR1*	*LMCD1*	rs9831647
ADAM17	*CORIN*	*IL10*	*MME*	*TGFBR2*	*LPL*	rs1800590
ADAM19	*CTLA4*	*INHA*	*NODAL*	*TGFBRAP1*	*LPL*	rs268
ADAM28	*DEF6*	*INHBA*	*NOS3*	*TIE1*	*near IL-10*	rs1800871
ADAM8	*DGKE*	*INHBB*	*NOTCH2*	*TIE2*	*near IL-10*	rs1800896
ADAM9	*EDN1*	*INHBC*	*PDGFD*	*TNF*	*NOS3*	rs61722009
ADAMTS7	*EDN2*	*INHBE*	*PDXDC1*	*VEGFA*	*NOS3*	rs2070744
ADM	*EHD3*	*IP6K1*	*PGF*	*VEGFB*	*NOS3*	rs1799983
ADM2	*EHD4*	*ITGA2*	*PSG11*	*VEGFC*	*VEGFA*	rs3025039
AGT	*ENG*	*ITGB1*	*ROCK1*			

4.4.8 Epidemiological study of *FLT1* variants (Study IV)

Two SNPs within *FLT1* that were found to have the strongest associations to pre-eclampsia were also compared to predefined epidemiological diagnoses derived from national health care registries. The analysis used imputed genotypes of 11,257 women from the FINRISK population cohort using a joint panel of Finnish whole genome sequences and 1000 Genomes phase I reference with a high imputation confidence (info metric > 0.97) (1000 Genomes Project Consortium *et al.* 2012, Chheda *et al.* 2017). Logistic regression (SNPTEST v2.5.2, EM algorithm) was used as the preliminary association test which was further confirmed with Fisher's exact test given the rarity of variants and several diagnoses. No multiple testing corrections to estimates were performed due to the explorative nature of this part of the analysis. Individuals with heart failure were identified using codes I50, 4289X, and 42700 in Finnish ICD-10, ICD-9, and ICD-8, respectively.

4.5 Bioinformatic methods

Sequencing primers were designed using Primer 3 and GenomeTester softwares (Rozen & Skaletsky, Andreson *et al.* 2006). The discovered single nucleotide polymorphisms were verified using SNPper and NCBI databases (Riva & Kohane 2002). VEP, HSF 2.4.1 and rnaSNP were used for in silica functional studies of intronic studies (Desmet *et al.* 2009, McLaren *et al.* 2010, Sabarinathan *et al.* 2013). Additionally, SIFT and PolyPhen2 were used to predict functional consequences of exonic SNPs (Kumar *et al.* 2009, Adzhubei *et al.* 2010).

4.5.1 Statistical methods (Studies I-IV)

Basic statistics including fisher's exact, χ^2 and independent-samples *t*-test to compare means of clinical characteristics between patients and controls were done using IBM SPSS statistics version 22 (IBM corp.)

In image quantification, an independent-samples *t*-test for analysing the significance of differences between means of values obtained from patient groups and controls was carried out for key statistical parameters including sum and mean. Same steps were repeated for the high-intensity analyses. Log_{10} transformation was used to normalise the image capture data. Normality was controlled by Shapiro–Wilk *W* test. (Study I)

Fisher's exact test was used to analyse for differences between segregation of *C4* deficiencies between groups of patients, and independent-samples *t*-test was used to assess the association of *C4* deficiencies, and immunohistochemistry fluorescence sum and mean values. (Study I)

Plink software was used in genetic association analyses (Purcell *et al.* 2007). Additional association analyses including 10 000 x permutation testing and haplotype analysis were conducted in Haploview (Barrett *et al.* 2005). Data were analysed using PlinkSeq, Plink (Purcell *et al.* 2007) and R. Kaviar (Glusman *et al.* 2011), SNPper (Riva & Kohane 2002) and VEP Build 37 was used in additional annotations (McLaren *et al.* 2010). Loss of function (LoF) analyses were conducted for each gene with associating variants *in silico* by the Loss of Function – tool [c]. he following annotations were calculated: LoF score < 0.2 is probably damaging, LoF score 0.2-0.7 is possibly damaging, LoF score < 0.7 is benign.

In candidate complement gene studies, MAF and HWE values were observed for cases and controls separately, and also separately for samples from different cohorts. Sites with >5% missing data or departure from Hardy-Weinberg equilibrium in controls ($p<0.05$) resulted in exclusion of the variant from association analyses (Studies II and III). In Sequenom genotyping data for Study III, 11 individuals were removed due to >10% failed genotyping. Three individuals were excluded due to unresolved discrepancy in genotyping results.

Study IV combined data from two cohorts and several genotyping methods. Quality control before meta-analysis included removal of singleton and monomorphic variants, removal of sites with >10% missing data in the targeted sequencing or a significant departure from Hardy-Weinberg equilibrium in controls ($p<0.001$). The combined dataset was analysed using Fisher's exact test of genetic association. The analysis was carried out in two phases: low frequency and rare variants (MAF<10%) and common variants (MAF>10%) were analysed separately. After quality control, 622 variants (443 variants with MAF<10% and 179 variants with MAF>10%) were included in the combined primary association analysis. Of these, 201 were in considered to be in putatively functional categories (missense, nonsense, or splice region variants) and 421 were in likely benign categories (synonymous, intronic, and intergenic). (Study IV)

From results of the association test, the two SNPs with strongest associations were further compared to 672 predefined diagnoses derived

from national health care registries using imputed genotypes of 11,257 women from the National FINRISK cohort. Genotypes were imputed using a combined panel of Finnish whole genome sequences and 1000 Genomes phase I reference with a high imputation confidence (info metric > 0.97) (1000 Genomes Project Consortium *et al.* 2012, Chheda *et al.* 2017). Associations were calculated using logistic regression (SNPTEST v2.5.2, EM algorithm) and then further confirmed with Fisher's exact test given the rarity of variants and several diagnoses. Due to the explorative nature of this part of the analysis, no multiple testing corrections to estimates were performed. (Study IV)

4.5.2 Relative extended homozygosity haplotype (Study III)

The discovered haplotype was subjected to Relative Extended Homozygosity Haplotype (EHH) based tests using REHH program. The analysis determines the evolutionary selection pressures contributing to the extent of the haplotype. REHH analysis uses the ancestral allele of a tagging SNP, here rs2287847 (Gautier & Vitalis 2012). REHH was conducted in R following the developer's instructions to compare extent of the associated haplotype in cases and controls.

4.6 Imaging (Study I)

Fluorescent microscopy images were captured using Olympus DP Manager (ver. 2.2.1.195) and Olympus DP Controller (ver. 2.2.1.227) image capture softwares with Olympus BX51 fluorescence microscope camera.

ImageJ 1.46 and Fiji-win32 software packages were used in immunofluorescent image quantification analyses (Abramoff *et al.* 2004). Background autofluorescence commonly introduces false positive readings to immunofluorescence images, so in order to correct for autofluorescence, using negative controls as samples, mean intensity +1 SD ($\overline{X}+\sigma$) was determined to be 7 at 20 ms exposure and 15 at 50 ms exposure times. Using an image specific zero threshold, several parameters of fluorescence intensity were recorded to differentiate between patterns of fluorescence as well as intensity. Sum was defined as mean intensity * area of positive fluorescence in pixels ($\overline{X}*\sigma$).

The high-intensity area quantification used a black-and-white rendering of the original fluorescent image. Using the black-and-white image as a reference, a threshold was set at 75% positive fluorescence, where after the percentage and pixel count of the positive area is used as a quantification to be compared between patient groups.

For quantification of the areas of most interest i.e. areas of high-intensity fluorescence, the top 75% proportion of fluorescence histogram was determined for each image using ImageJ 1.46 software and the calculated maximum fluorescence value. The calculated 75% minimum value was used as a cut-off point creating representative and comparable images of high-intensity regions. Thereafter, Pearson-correlation of high-intensity proportions across different stainings was calculated independently for each patient group. Positive correlations were interpreted as two components being observed in the same sample, while negative correlations represent two components not co-occurring on the same sample.

5. RESULTS AND DISCUSSION

Complement system is a key contributor to development of pre-eclampsia. Excess complement activation causes and aggravates sterile inflammation and endothelial dysfunction (Hertle *et al.* 2014), which is typically seen in pre-eclampsia. The role of complement mediated inflammation seems to be particularly prominent in severe pre-eclampsia (Burwick *et al.* 2013). Deposits of complement component C4 have been observed in renal biopsies of pre-eclamptic women providing a link between complement activation and renal dysfunction, an important diagnostic characteristic of pre-eclampsia (Penning *et al.* 2015). Other immunological mechanisms with adverse consequences for the systemic endothelium may include neutrophil extracellular trap (NET) formation by neutrophils as a consequence of activation of maternal leukocytes (Laresgoiti-Servitje 2013).

Inflammation during pre-eclampsia has varied systemic consequences. Endothelial dysfunction may be caused by shedding of foetal trophoblast membranes from the syncytium of the pre-eclamptic placenta (Smarason *et al.* 1993). Oxidative stress and inflammation among other mechanisms interact to contribute to endothelial dysfunction (Steyers & Miller 2014).

The results of this study combine case-control data from candidate gene studies using several methods of sequencing and immunohistochemistry to pinpoint association to pre-eclampsia in the Finnish population. We have explored genes of complement components C3, C4, and CD46. Additionally, we studied localisation and quantity of ten components of the complement system and the anti-angiogenic factor sEng in placentae from pre-eclamptic pregnancies and non-pre-eclamptic women. Finally results from candidate genes from several biological pathways known to associate with pre-eclampsia were studied in a targeted exome sequencing study. Complement components were the subject of Studies I-III, anti-angiogenic factors were studied in Studies I and IV, and other candidate genes in Study IV. Before the results are discussed topic-wise, the respective study is indicated in parentheses next to the heading.

5.1 Complement genotyping (III)

We hypothesised that genetic variants within genes coding for the complement system may associate to pre-eclampsia. As complement system is comprised of 40 activating and regulating components, which are coded for by at least 41 genes (total C4 is coded by two genes: *C4A* and *C4B*), we approached the hypothesis by selecting previously described loci within key components of the complement system to pinpoint a possible gene of interest for closer study.

Seventeen genes coding components of the complement system or known candidate genes for pre-eclampsia were genotyped for selected SNPs using the Sequenom platform. In 64/72 SNPs, no differences were observed between 259 pre-eclamptic women and 426 non-pre-eclamptic controls. 72/93 SNPs had minor allele frequency of >0.05. These were included in the analyses. 685/720 individuals had genotyping success of >90% and these were included in the analyses. Of the analysed SNPs listed in Table 6, preliminary analyses revealed possible associations in Ficolins 1 and 2, Factor H, C3 and Factor B (data not shown). The sample size available at the time the study was initiated limited the power of our analysis. It is possible that true associations within the remaining genes may be discovered using a larger sample size of well characterised patients and controls. When association analyses were controlled for multiple testing, the most significant results mapped to *C3*. The permutation procedure reduced the significant p-values to two SNPs in *C3* (rs2230205; p=0.032 and rs2230204; p=0.005), and therefore only results concerning *C3* are discussed hereafter.

Two haploblocks with significant association to pre-eclampsia were discovered in C3 by experimental haploblock creation of three neighbouring SNPs (Table 7). In conditional haplotype analyses between rs2230204 and rs2230205 the previous was found most likely to have independent effect ($\chi 2=5.1$, df=1, p=0.0239). Rs2230204 and rs22302045 are synonymous SNPs located in exon 14 of the C3, in the middle of the gene. They were found to be in linkage disequilibrium. To explore the genetic architecture of C3 and look for possible associating variants to severe pre-eclampsia, the exons and selected introns as well as regulatory regions were sequenced in patients with severe pre-eclampsia and non-pre-eclamptic controls using Sanger sequencing and Sequenom iplex.

Table 6. The analysed polymorphisms listed by chromosome, gene symbol and rs identifier.

Chromosome	Gene	RSID	Chromosome	Gene	RSID
1	*MASP2*	RS2273347	9	*FCN2*	RS17549193
1	*MASP2*	RS1033638	9	*FCN2*	RS7851696
1	*MASP2*	RS1782455	9	*FCN1*	RS1071583
1	*MASP2*	RS12711521	9	*FCN1*	RS2274845
1	*MASP2*	RS3765900	9	*FCN1*	RS2989722
1	*FCN3*	RS4970521	9	*FCN1*	RS10858293
1	*FCN3*	RS10794501	9	*FCN1*	RS10117466
1	*THBS3*	RS35154152	9	*FCN1*	RS10120023
1	*THBS3*	RS914615	9	*FCN1*	RS2989727
1	*CFH*	RS800292	10	*MBL2*	RS5030737
1	*CFH*	RS1061147	10	*MBL2*	RS1800450
1	*CFH*	RS2274700	10	*MBL2*	RS7096206
1	*CFH*	RS1329428	10	*MBL2*	RS11003125
3	*MASP1*	RS698090	11	*SERPING1*	RS3758918
3	*MASP1*	RS698092	11	*SERPING1*	RS1005510
3	*MASP1*	RS3105782	11	*SERPING1*	RS1557522
3	*MASP1*	RS1357134	11	*SERPING1*	RS4926
3	*MASP1*	RS850307	13	*FLT1*	RS9554314
3	*MASP1*	RS6783637	13	*FLT1*	RS7326277
3	*MASP1*	RS7624953	13	*FLT1*	RS7993418
5	*THBS4*	RS3813667	13	*FLT1*	RS2296189
5	*THBS4*	RS423906	15	*THBS1*	RS2228261
6	*CFB*	RS641153	15	*THBS1*	RS2292305
6	*CFB*	RS537160	19	*C3*	RS17030
6	*CFB*	RS541862	19	*C3*	RS10402876
6	*CFB*	RS4151658	19	*C3*	RS423490
6	*CFB*	RS2072633	19	*C3*	RS366510
6	*THBS2*	RS7382711	19	*C3*	RS2230205
6	*THBS2*	RS12178180	19	*C3*	RS2230204
9	*FCN2*	RS3124952	19	*C3*	RS1047286
9	*FCN2*	RS3811140	20	*THBD*	RS1042580
9	*FCN2*	RS7865453	20	*THBD*	RS1042579
9	*FCN2*	RS17514136	23	*CFP*	RS1048118
9	*FCN2*	RS3128624	23	*CFP*	RS8177079
9	*FCN2*	RS7037264	23	*CFP*	RS909523
9	*FCN2*	RS12684723			

Table 7. Two experimental haploblocks with predisposing and protective associations to pre-eclampsia. F_A: frequency of haplotype in affected individuals, F_U: frequency of haplotype in unaffected individuals, df-degrees of freedom.

Haplotype	F_A	F_U	χ^2	df	P-value	SNPs	Effect
GGT	0.181	0.235	5.707	1	0.017	RS10402876|RS423490|RS366510	protective
CGT	0.415	0.345	6.634	1	0.010	RS10402876|RS423490|RS366510	predisposing
CTC	0.279	0.352	7.87	1	0.005	RS2230205|RS2230204|RS1047286	protective
TCC	0.229	0.181	4.644	1	0.031	RS2230205|RS2230204|RS1047286	predisposing

5.2 C3 Sequencing (III)

The results from above described complement gene study led us to the hypothesis that genetic variants within the *C3* may associate to pre-eclampsia. To explore this hypothesis, sequencing of *C3* was done using Sanger sequencing and Sequenom iplex. C3 is the central component of complement activation. Unfunctioning C3 or complete deficiency of the protein increases susceptibility to encapsulated pyogenic bacteria being potentially fatal and therefore rare (Lokki & Colten 1995). There are less than 30 confirmed cases of *C3* deficiency and interestingly several cases with gain-of-function mutations described worldwide (Grumach & Kirschfink 2014). However, variants in C3 associate to autoimmune diseases and aHUS among other diseases.

Sanger sequencing of C3 exons, flanking regions, and selected introns revealed a functional haplotype of 16 SNPs spanning the functionally critical sections in the middle of the gene. The region covered by the haploblock on the protein structure is indicated by blue square in Figure 9. Rs423490 is included in the experimental haploblock associating to, and protecting from pre-eclampsia (Table 7) as well as in the actual haplotype discovered by Sanger sequencing (Table 8), but Sanger sequencing did not reveal a single SNP association at this locus. The three SNPs with most robust associations to severe pre-eclampsia are also located within the haploblock further supporting its functional significance.

Regulatory regions, such as promoter or the pre-promoter microsatellite region known to associate to temporal lobe epilepsy and seizures did not yield significant associations to pre-eclampsia (Jamali *et al.* 2010). However, these results should be treated suggestive rather than conclusive due to small sample size in these analyses.

Table 8. Summary of findings in complement component 3 gene sequencing project. The p- values in parentheses are not conclusive due to small MAF and/or sample size in this location. Only statistically significant ($p<0.05$) p-values indicating association to pre-eclampsia are shown.

Genomic position (GRCh38)	RSID	Protective p-value= 0.044	Predisposing p-value= 0.011	Predisposing SNP, p-value
6677978	rs17030			
6679349	rs344555			
6681980	rs7951			
6682103	rs45532534			
6686410	rs11666133			
6686493	rs2241391			(0.044)
6686567	rs11665922			
6686648	rs237554			
6690602	rs3745568			
6690629	rs375600369			
6693152	rs10414623	C	C	
6693229	rs2241390	C	T	
6693262	rs11569492	C	T	
6693306	rs2241389	C	T	
6693376	rs389404	C	T	
6694388	rs385791	C	C	
6696331	rs2287848	C	T	0.041
6696485	rs2287847	G	G	
6696546	rs2287846	C	G	
6696586	rs2287845	T	C	0.038
6697395	rs423490	C	C	
6697611	rs77063881	G	G	
6697818	rs366510	A	C	0.039
6702146	rs428453	C	G	
6702235	rs432823	G	A	
6702444	rs11569450	C	C	
6702489	rs200046246			
6702587	rs406514			
6707655	rs558232596			
6707902	rs144432231			
6709693	rs2230205			
6709837	rs2230204			
6710771	rs2230203			
6710937	rs10411506			
6713164	rs11085197			
6713251	rs1047286			
6713280	rs2230201			
6714024	rs11569571			
6718376	rs2230199			
6719442	rs190390034			(0.005)
6720709	rs11569569			
6720961	rs183805948			(0.017)
6721227	rs146998274			

Using the ancestral allele of SNP rs2287845, the extent of selection pressure on the haploblock structure was evaluated using the REHH analysis. The analysis revealed that the controls hold tighter haploblock structures than women with severe pre-eclampsia. The tight haploblock suggests that the structure is indeed due to a positive evolutionary selection pressure. Selection pressures were slightly relaxed in pre-eclamptic women suggesting a loosened force of active selection. It is possible that the observed selection pressure in pre-eclamptic women is the force of selection past, i.e. remnants of the selection pressure previously active on the haplotype. It is also possible that the genetic haplostructure of severe pre-eclamptics reflect a distinct selection pressure due to thus far unknown fitness advantage of this haplotype.

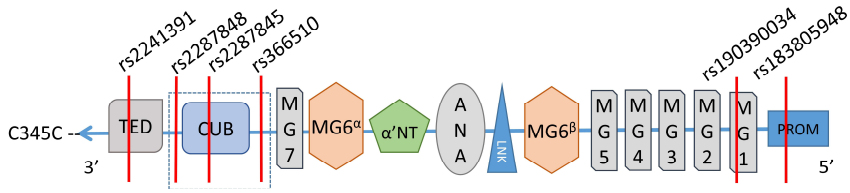

Figure 9. The C3 domain structure in relation to the associating single SNPs. Together with CUB (complement C1r/C1s, Uegf, Bmp1) and TED (thio-ester containing) domains, the α'NT forms the functional region containing the critical binding sites for factor B as well as inhibitors CR1 and factor H causing activation or deactivation of C3. The extent of the associating haplotype is shown in the blue square.

The mechanism of haplotype association to severe pre-eclampsia is unclear, but the effect may be due to functional or regulatory attributes of this region. Functional changes in C3 may affect the extravillous trophoblasts' capacity to evade complement activation by C3 binding thereby compromising deep placentation and spiral artery remodelling. Excess complement activation may also be involved in a later stage of pre-eclampsia influencing severity of symptoms such as inflammation and hypertension (Lillegard *et al.* 2013).

The results of this study indicate that parallel to mice, C3 also plays a central role in the healthy pregnancy in humans (Xu *et al.* 2000). The

complement system has been indicated in some cases of a related condition, HELLP syndrome, a rare condition which is considered to be a complication of severe pre-eclampsia. By the inhibitor drug treatment eculizumab, which targets C5, a patient suffering from HELLP was able to extend her pregnancy until a safer time to secure a healthy delivery (Burwick & Feinberg 2013). The results of this study indicate that an earlier point within complement activation pathways, C3 may also be a plausible target for drug development. With further confirmation of these results, a combination of C3 polymorphisms may also be incorporated into a genetic predictor tool to detect those patients who are most at risk of developing severe pre-eclampsia.

5.3 Complement in placenta (I)

We hypothesized that excess or unregulated complement activation may be detectable within the placenta tissue of pre-eclamptic patients. Furthermore, localisation and quantity of complement components in relation to tissue integrity in a healthy and pre-eclamptic placenta may be observed in the placenta by immunohistochemistry. Nine components of the complement system were studied alongside with sEng to pinpoint localization of complement components in the placenta tissue and to assess, whether their quantity or localisation is associated to pre-eclampsia. The studied components were selected to represent the key activators and regulators of components from classical (C1q, C4c, C4bp, DAF, and MCP), alternative (C3c, C3d, FH, MCP), and terminal (C9 and CD59) pathways of complement activation. C3c was considered to indicate a general indicator of complement activation, while C3d was considered to be an indicator of chronic and specific C3 deposition on the placenta tissue.

Complement components were visualised on the placenta tissue using immunofluorescence staining of placental sections from pre-eclamptic and healthy pregnancies. Intensity of fluorescence was measured by sum amount as well as mean of total intensity. Associations of intensity readings between groups are given in table 9 according to the component where association was discovered. Furthermore, the high intensity areas thought to represent the functionally most significant areas of complement deposition, were correlated against each other and significant correlations between groups are given in table 10. Negative result of Pearson's r signifies that in the given group, the two components

are negatively correlated, while positive results signify that the more there is of one component, the more there will be of the second one.

Table 9. Significant associations of t-test analysis between diagnostic groups. Both mean and sum of intense fluorescence areas were analysed and compared against all diagnostic groups. Early – early onset pre-eclampsia, late – late onset pre-eclampsia, PE – pre-eclampsia, s-Endoglin – soluble endoglin, C1q – complement component 1q, df – degrees of freedom.

Association	Test	t-test	p-value	df
C1q				
early/late	Sum	2.273	0.046	10
early/late	Mean	3.614	0.005	10
late/control	Sum	2.793	0.015	13
late/control	Mean	2.993	0.01	13
s-Endoglin				
PE/control	Sum	2.008	0.059	19
PE/control	Mean	2.463	0.023	19
early/control	Sum	1.524	0.150	14
early/control	Mean	1.815	0.091	14
late/control	Sum	1.842	0.090	12
late/control	Mean	2.825	0.015	12

S-Eng was used as a positive control in this study, because it is known to stain circumferentially on the placenta (Gu *et al.* 2008). Furthermore, s-Eng is increased in pre-eclampsia in comparison to healthy pregnancies (Venkatesha *et al.* 2006). Due to small sample size and inaccuracy of the semi-quantification protocol, we were not able to corroborate the correlation between s-Eng and pre-eclampsia severity (Leanos-Miranda *et al.* 2017), but our results are in accordance with other studies who did not differentiate late- and early-onset pre-eclampsia by levels of endoglin expression (Sitras *et al.* 2009, Nishizawa *et al.* 2007). The significant association between s-Eng deposition in pre-eclamptic and controls in Table 9 (mean p-value=0.023) proves that the semi-quantification of

immunohistochemistry stainings may reveal trends in placental deposition and allows for comparison between pre-eclamptics and controls.

The association of C1q deposition in Table 9. between early-onset and late-onset pre-eclampsia and on the other hand between late-onset pre-eclampsia and controls reveals the role of complement-mediated housekeeping function in pre-eclampsia. It is likely, that activation of classical pathway of complement system signifies here the increase in demand for debris clearance which is associated with break-down of syncytiotrophoblast integrity, which is typical in pre-eclampsia. In our data, this is particularly seen in the case of late-onset pre-eclampsia, where C1q is most abundant. In conclusion, the association between complement and pre-eclampsia here is most likely due to the effects of the disease rather than being the underlying cause of it. That being said, activation of complement pathways will serve to induce and maintain inflammatory response in the placenta.

Correlation between quantification results of different complement components in the same placentae point were used to point us towards the active complement pathways. When a negative correlation between an activator of an early pathway and an activator of a terminal pathway component is observed, it is interpreted as successful regulation of complement activation.

The role of the complement in pre-eclamptic and control placentae is further clarified by the significant correlations in Table 10. The more C3b deposition there is, the more endoglin is also observed indicating an association between C3 and early-onset pre-eclampsia. Controls typically have more of the membrane bound inhibitors DAF and MCP, but this correlation is absent in pre-eclamptic placentae. Interestingly, in late-onset pre-eclampsia the activation of classical pathway (C4) is correlated with activation of terminal pathway (C9) even in the presence of strong correlation between the soluble regulator C4bp and C4. Another indication of classical pathway in the late-onset pre-eclampsia is the negative correlation between C1q and soluble regulator FH. FH is known to bind independently and compete with C1q in apoptotic surfaces and debris (Kishore & Sim 2012).

Table 10. The significant correlations of complement component among diagnostic groups (PE - pre-eclampsia).

Diagnosis	Component 1	Component 2	Pearson's r	p-value (2-Tailed)
Classical pathway				
Early-onset PE	C1q	C9	−0.878	0.009
Early-onset PE	DAF	C4	0.824	0.023
Late-onset PE	C4	C9	0.921	0.026
Late-onset PE	C4bp	C4	0.962	0.009
Control	C4bp	MCP	−0.659	0.038
Alternative pathway				
Early-onset PE	C3b/iC3b	s-Eng	0.829	0.021
Control	FH	C9	0.671	0.034
Classical and alternative pathways				
Late-onset PE	C1q	FH	−0.932	0.021
Control	DAF	MCP	0.634	0.049

The results of this study indicate that components of the complement system are present in a specific pattern on the placenta. The most typical pattern of localisation was circumferentially on the syncytiotrophoblast, which is effectively the maternal-foetal interface (data not shown).

Placenta during pregnancy must be carefully protected by inhibitors of complement activation from complement mediated tissue destruction and cell death (Buurma *et al.* 2012). An example of failed complement regulation is shown in an immunofluorescent overlay staining of a paraffin fixed placental sample in Figure 10. In comparison of the images, it is observed that in the region, where coverage of FH is not adequate, complement activation likely through amplification loop (green in panel B) causes the syncytiotrophoblast to break down and tissue deformation occurs.

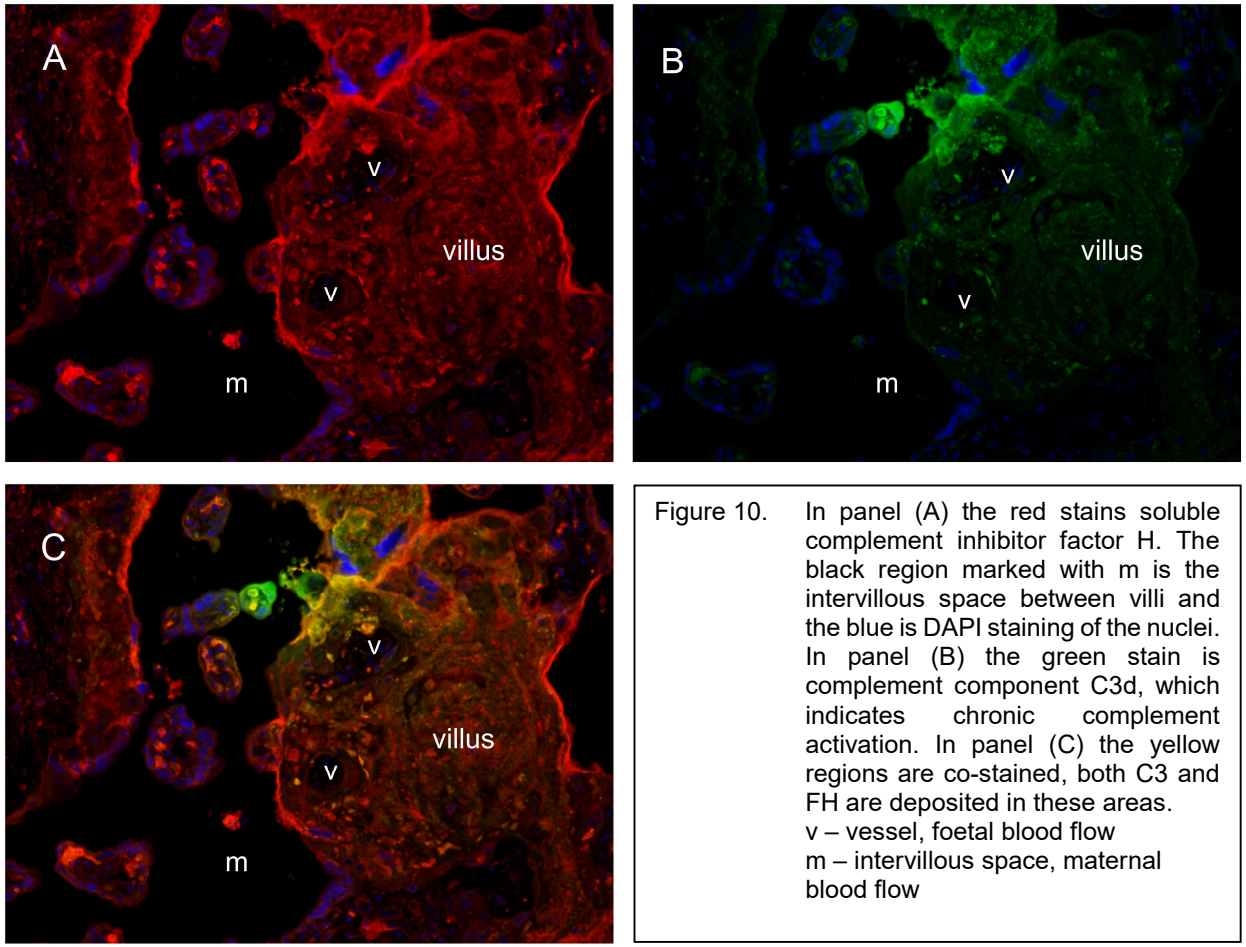

Figure 10. In panel (A) the red stains soluble complement inhibitor factor H. The black region marked with m is the intervillous space between villi and the blue is DAPI staining of the nuclei. In panel (B) the green stain is complement component C3d, which indicates chronic complement activation. In panel (C) the yellow regions are co-stained, both C3 and FH are deposited in these areas.
v – vessel, foetal blood flow
m – intervillous space, maternal blood flow

The results and study design from Study I are summarised in Figure 11. The localisation and pattern of complement deposition differ between pre-eclamptic and control placentae, but we cannot conclusively differentiate between functional differences in different diagnostic groups, or the effect of tissue disruption, known to be associated with pre-eclampsia, on the pattern of complement component deposition. The correlation of C9 indicating MAC deposition with the classical pathway activating components C4 in the patient groups may be indicative of breakdown of complement regulation and/or uncontrolled classical pathway activation in pre-eclampsia. In controls, the soluble regulator factor H, correlated with C9 indicating a healthy regulation of complement activation in these placentae. Furthermore, the control placentae are well protected by correlating amounts of surface-bound regulators DAF and MCP.

5.4 C4 deficiencies in pre-eclampsia (I)

Complement component C4 is coded by two genes, *C4A* and *C4B*, with partially overlapping functions. Typically, an individual may have between zero and four copies of both genes, although zero copies of both genes resulting in complete *C4* deficiency are very rare (Lokki & Colten 1995). This study was based on the hypothesis that the copy number variation observed within the genes coding for C4 may associate to pre-eclampsia. Results of this study suggest that deficiencies in *C4* may predispose to pre-eclampsia. *C4A* or *C4B* deficiencies were found almost twice as often in early-onset pre-eclampsia patients than in healthy controls (Table 11). *C4A* deficiencies are observed in 16% of general population in Finland (Wennerstrom *et al.* 2013). *C4A* deficiencies were found in 40% (2/5) of pre-eclampsia mothers with late-onset disease and 43% (3/7) of pre-eclampsia mothers with early-onset disease. None were observed in the controls (n=7). Because of the small number of samples, the difference was on the borderline statistically significant (p=0.055 Fisher's exact two-sided test). Only two individuals had total *C4* deficiency (total *C4A* deficiency in one control mother and total *C4B* deficiency in one child born from early-onset pre-eclamptic pregnancy).

Table 11. Frequency of C4A and C4B gene deficiency in diagnostic groups and controls.

		PE (pooled) (n=12)	Early-onset PE (n=7)	Late-onset PE (n=5)	control (n=8)
C4A or C4B deficiency	maternal	0.667	0.714	0.600	0.375
	foetal	0.700	0.667	0.750	0.500
C4A deficiency	maternal	0.417	0.429	0.400	0
	foetal	0.300	0.333	0.250	0.375
C4B deficiency	maternal	0.333	0.286	0.400	0.375
	foetal	0.400	0.333	0.500	0.250

The genetic analyses in Study I showed that *C4A* deficiency was more frequent in pre-eclamptic women than controls. Although the results were not conclusive due to small sample size, it would seem that *C4* deficiencies are more of importance in maternal than foetal genotype. Furthermore, the copy number of *C4* seems to decrease with the severity of pre-eclampsia symptoms. *C4A* deficiencies have previously been linked to autoimmune diseases (Samano *et al.* 2004). The patients in this study did not suffer from diagnosed autoimmune diseases. The high incidence of *C4A* deficiency in pre-eclampsia supports the importance of classical pathway of complement system in pre-eclampsia.

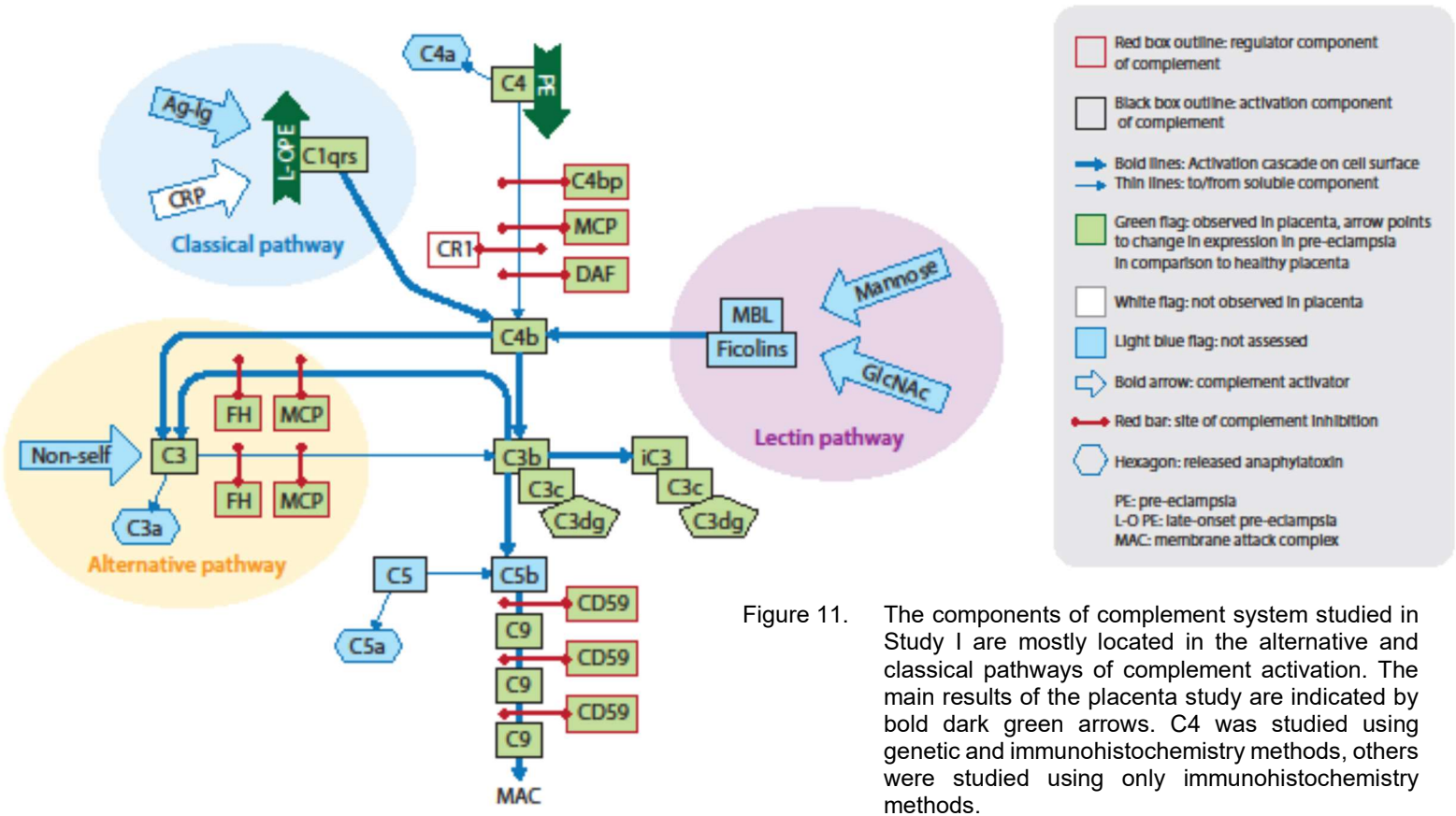

Figure 11. The components of complement system studied in Study I are mostly located in the alternative and classical pathways of complement activation. The main results of the placenta study are indicated by bold dark green arrows. C4 was studied using genetic and immunohistochemistry methods, others were studied using only immunohistochemistry methods.

5.5 Sequencing of MCP (CD46) (II)

MCP has the capacity to regulate both alternative and classical pathways of complement activation by binding to C3b or C4b, and acting as a co-factor to the inactivator enzyme Factor I. MCP is a widely expressed type 1 membrane bound protein. The gene *MCP* is located in the regulators of complement activation (RCA) gene cluster in chromosome 1 in proximity of genes for numerous other complement inhibiting components (Holers *et al.* 1985, Liszewski *et al.* 1991). In this study, fifteen SNPs were discovered, including three novel variants. The SNPs are listed in Table 12 according to their location on the gene. None of the SNPs were associated to pre-eclampsia with severe proteinuria.

A disease of the kidney, aHUS, can be caused by mutations in the *MCP* (Manchester *et al.* 1995, Richards *et al.* 2003, Fremeaux-Bacchi *et al.* 2006, Fang *et al.* 2008). Proteinuria is one of the defining symptom of pre-eclampsia, but its extent can vary 10-fold between patients. For this project, pre-eclamptic women with severe proteinuria were selected, because renal dysfunction due to uncontrolled complement activation was suspected to be the underlying link between pre-eclampsia and kidney diseases.

To investigate whether sequence variations in the *CD46* promoter, splice sites and exome might predispose Finnish women to pre-eclampsia we sequenced these areas within the *MCP* gene in pre-eclamptic women with severe proteinuria and in non-pre-eclamptic controls. A larger cohort of controls was used in this study, to increase the power of the analysis in the relevant region of the gene, because A304V (rs35366573) was previously shown to be associated with pre-eclampsia (Salmon *et al.* 2011).

The results of this study do not corroborate the previously reported association of A304V to severe pre-eclampsia (Salmon *et al.* 2011). We observed a high MAF of approximately 6% in both cases and controls and we also found one control individual, who was homozygous to 304*V. The other functional SNP K32N (rs150429980) located in CCP 1 was found in one heterozygous case and one heterozygous control and thereby results are inconclusive.

Table 12. Fifteen single nucleotide polymorphism (SNPs) discovered in Sanger sequencing of membrane cofactor protein gene (MCP). The number of individuals that passed quality control is given in N cases and N controls.

	MAF cases	MAF controls	N cases	N controls	Function
Promoter					
rs2796268	0.453	0.457	95	94	regulatory
rs41266397	0.069	0.074	95	95	regulatory
Exonic					
rs150429980	0.005	0.005	95	94	ex2 / K32N
rs12126088	0.011	0.000	95	94	ex4 / synonymous
rs41258244	0.069	0.074	95	94	ex5 / synonymous
rs35366573	0.058	0.061	95	188	ex11 / A304V
Intronic					
rs2724374	0.247	0.200	95	95	close to ex8
NewSNP1	0.000	0.005	92	97	close to ex13
NewSNP2	0.005	0.000	95	94	cytoplasmic tail
NewSNP3	0.005	0.000	95	187	cytoplasmic tail
rs7144	0.452	0.457	95	94	cytoplasmic tail
rs14374	0.053	0.021	95	95	cytoplasmic tail
rs185457983	0.000	0.005	95	94	cytoplasmic tail
rs193023975	0.005	0.000	95	94	cytoplasmic tail
rs1237	0.069	0.042	95	95	cytoplasmic tail

It is possible, however, that MCP plays a part in a particular subtype of pre-eclampsia, due to the heterogeneous nature of the disease. Regulators of complement activation may be particularly important on the foetal tissue, but also in controlling inflammation and preventing tissue destruction on the placental bed. Severe pre-eclampsia has been previously linked with elevated levels of alternative pathway activation in the mother (Hoffman *et al.* 2014).

5.6 Maternal FLT1 variants protect from pre-eclampsia (IV)

Dysregulation of angiogenesis is among the most promising hypotheses of study to the pre-eclampsia pathophysiology over the last 15 years (Levine *et al.* 2004). A high ratio of sFlt1 to PlGF is considered a promising biomarker for prediction of pre-eclampsia (Villa *et al.* 2013, Sunderji *et al.* 2010, Sahai *et al.* 2017). We hypothesised that genetic variants in the *FLT1* may associate to pre-eclampsia.

We found four SNPs to be associated to pre-eclampsia in the *Flt1* (Table 13). Two of the variants, rs35832528 and rs141440705, were protective while the others, rs148588802 and rs61763183, were predisposing. Rs148588802 is rare, and rs61763183 is associated at a borderline level only, so these results should be treated with caution.

Among the observed associating variants in *FLT1*, rs141440705 causing R54S is more likely of functional importance, because it results in a polar change (positive->neutral) within the immunoglobulin -like domain 1, one of the functional domains of the protein (Christinger *et al.* 2004). On the other hand, rs141440705 is located in the last nucleotide of exon 3. Therefore, the variant potentially affects splicing as well as the coded amino acid sequence.

In study IV we provide further evidence on the link between pre-eclampsia and heart failure. The results suggest that those *FLT1* variants that protect from pre-eclampsia also protect from heart failure (Table 14).

The anti-angiogenic *FLT1* is a tempting candidate gene for the suspected link between pre-eclampsia and increased risk for CVD in later life. *FLT1* has been indicated in coronary artery disease in a genome wide

association study as well as in a follow-up study indicating association to long-term cardiovascular events (Lee *et al.* 2013, Lee *et al.* 2016). The circulating sFlt1 levels are higher in pregnant, in comparison to non-pregnant women. From serum, increased levels of sFlt1 are indicated in both pre-eclampsia and in peripartum cardiomyopathy. In both diseases, high levels of sFlt1 correlate with severity of symptoms (Damp *et al.* 2016). Pre-eclampsia may increase the risk of peripartum cardiomyopathy (Hilfiker-Kleiner *et al.* 2015). It has been suggested that sFlt1 may be toxic to the heart thereby contributing to the development of peripartum cardiomyopathy in susceptible women (Shahul *et al.* 2016). In heart failure during pregnancy, extreme sFlt1 levels in at least the 95th percentile have been reported (Rhee *et al.* 2011). Independent from pregnancy, heart failure after myocardial infarction is also reflected in extreme levels of sFlt1 (Onoue *et al.* 2009).

In a meta-analysis of results from seven studies, it was shown that an increase of 3.6-fold in risk of heart failure is associated with a history of pre-eclampsia, predominantly during 1-10 years after pre-eclampsia (Wu *et al.* 2017). Cardiac dysfunction in predisposed pre-eclamptics is likely mediated by antiangiogenic factors (Shahul *et al.* 2016). High sFlt1 is also linked to impaired renal function, heart failure, and early mortality in patients with chronic kidney disease (Di Marco *et al.* 2015). The role of the sFlt1 in proteinuria and glomerular endotheliosis was established in the beginning of the last decade, but given the importance of proteinuria and hypertension to the diagnosis of pre-eclampsia, the specific mechanisms have received unexpectedly little attention in the last decade, while the role of sFlt1 in hypertension has been more widely studied (Maynard *et al.* 2003).

Further research is required to assess the interaction between genetic variants of *FLT1,* and their consequences to the development of the symptoms related to pre-eclampsia. Specifically, the immunological and renal effects of sFlt1 warrant further investigation. It is likely that future research will tie these effects to the widely reported increased risk of heart failure in later life. In support of this theory, the results of this study indicate that *FLT1* variants that protect from pre-eclampsia may also protect from heart failure. In susceptible patients, angiogenic imbalance may link pre-eclampsia to later life cardiovascular morbidity. The research to the possibility of blocking sFlt1 production or development of neutralising antibodies against angiogenic proteins may in the future provide therapeutic benefit for individuals with genetic predisposition (Thadhani 2010, Maynard & Karumanchi 2011). The results of this study add to the toolbox of genetic markers that may been soon used to determine a patient's risk of these serious diseases.

Table 13. Four variants of the fms related tyrosine kinase 1 gene (*FLT1)* are associated with pre-eclampsia. Non-adjusted p-values are shown.

RSID number	P-value	OR (95% confidence interval)	MAF in total sample	Count with minor allele $n_{cases}/n_{controls}$	Consequence (distance from exon, base pairs)
rs35832528	2.49E-4	0.387 (0.205 - 0.678)	0.010	14/122	missense variant, E982A
rs141440705	0.003	0.442 (0.233 - 0.779)	0.010	14/106	missense variant, R54S
rs148588802	0.038	10.328 (0.829 - 540.960)	0.002	3/1	intron variant (+40)
rs61763183	0.050	1.653 (0.967 - 2.754)	0.020	24/50	downstream gene variant (+73)

Table 14. E982A and R54S in fms related tyrosine kinase 1 gene *(FLT1)* protect from heart disease as examined across registry-derived disease endpoints.

RSID number	Amino acid change	P-value	OR	95% confidence interval	$n_{cases}/n_{controls}$
rs35832528	E952A	0.007	0.368	0.164-0.830	301/10956
rs141440705	R54S	0.007	0.340	0.140-0.826	297/10960

5.7 Candidate gene associations (IV)

We hypothesised that genes not coding for components of the complement system may also contribute to the patients' predisposition to pre-eclampsia, which is known to be a multifactorial disease. In addition to the *FLT1* association to pre-eclampsia described above, associating variants were found in twenty-three other candidate genes. The candidate genes were selected to represent key pathways that have a suspected link to pre-eclampsia. These include common immunological genes, genes with previously reported or suspected association to pre-eclampsia or related pregnancy complications, and genes linked to susceptibility to cardiovascular disease, or belonging to suspected pathways of importance such as angiogenesis. Genes coding for factors of the coagulation cascade and components of the complement system were excluded from this study to be later analysed separately. The significant results of the association studies are listed in Table 15. The most promising candidate genes with associating variants are discussed below.

Corin codes for a member of the type II transmembrane serine protease class of the trypsin superfamily that is predominantly expressed in cardiac tissue (Yan *et al.* 1999). Through cleavage of atrial natriuretic peptide (ANP) Corin regulates blood pressure (Yan *et al.* 2000, Li *et al.* 2017). Corin also likely plays an important role in the regulation of CVDs (Hooper *et al.* 2001). Recent evidence has shown that corin-ANP autocrine function in the kidney may regulate sodium homeostasis suggesting, that local corin function may also be an underlying cause in kidney diseases (Li *et al.* 2017). In pre-eclampsia, association with corin has been previously indicated with two population-specific variants having similar odds ratios of approximately 2.5 in intronic SNPs to what we found in exonic variants rs61759670 and rs61760500 (Stepanian *et al.* 2014). Corin may have an important role in trophoblast invasion and spiral artery remodelling (Cui *et al.* 2012, Kaitu'u-Lino *et al.* 2013) and immunohistochemistry stainings show over-expression in a subset of decidual cells from pregnancies with foetal growth restriction in comparison to healthy pregnancies (Miyazaki *et al.* 2016).

Activin A receptor type 1 (ACVR) and type 1C (ACVR1C) belong to the transforming growth factor signalling molecule receptor group. Previous results from rs13406336 and rs4556933 in *ACVR1* and *ACVR1C* have been inconclusive. They were studied as potential candidate SNPs, but no significant association to pre-eclampsia was established in a Norwegian population (Roten *et al.* 2009). The result in this study indicates a likely association to pre-eclampsia in these variants.

Table 15. The list of variants with possible associations to pre-eclampsia.
OR – Odds ratio. MAF – Minor allele frequency. Non-adjusted p-values are given.

Candidate gene	Gene name	P-value	OR (95% confidence interval)	MAF in total sample	Consequence (distance from exon, base pairs)	Primary pathway, gene function
rs61758484	*CORIN*	0.003	2.658 (1.320 - 5.261)	0.013	non-coding transcript exon variant, E10K	Cardiac conduction, myometrial relaxation/contraction
rs34106916	*ANGPTL1*	0.006	3.186 (1.325 - 7.597)	0.008	synonymous variant, Q103Q	Immunological cell signalling
rs61759670	*CORIN*	0.010	2.082 (1.147 - 3.693)	0.016	missense variant, Y907T	Cardiac conduction, myometrial relaxation/contraction
rs80338240	*JAG1*	0.010	0.256 (0.051 - 0.806)	0.004	intron variant (-11)	Notch signalling
rs147998709	*GPR98*	0.011	13.757 (1.360 - 675.490)	0.003	intron variant (+24)	Development of central nervous system.
rs2290843	*ADAM12*	0.011	0.726 (0.561 - 0.932)	0.071	synonymous variant, T326T	A disintegrin and metalloprotease domain-family, actin dynamics signalling pathway, fertilisation
rs61760500	*CORIN*	0.011	2.550 (1.179 - 5.382)	0.010	intron variant (+13)	Cardiac conduction, myometrial relaxation/contraction
rs147942437	*ADAM28*	0.012	5.181 (1.226 - 24.998)	0.004	missense variant, L449P	A disintegrin and metalloprotease domain-family

Candidate gene	Gene name	P-value	OR (95% confidence interval)	MAF in total sample	Consequence (distance from exon, base pairs)	Primary pathway, gene function
rs13406336	*ACVR1*	0.013	2.423 (1.129 - 5.062)	0.011	missense variant, A15G	Apoptosis pathway, cell signalling. Receptor for growth and differentiation factors. TGF-beta superfamily member.
rs142436579	*ADAM28*	0.014	0.366 (0.129 - 0.852)	0.005	missense variant, R219S	A disintegrin and metalloprotease domain-family
rs140437272	*INHBE*	0.014	0.477 (0.236 - 0.881)	0.013	missense variant, P27L	Peptide hormone metabolism, apoptosis, immune response. TGF-beta superfamily member.
rs201756397	*FLT4*	0.014	Inf (1.283 - Inf)	0.001	synonymous variant, E926E	Receptor for VEGFC and VEGFD. Lympahgiogenesis.
rs80069610	*GPR98*	0.016	2.499 (1.122 - 5.411)	0.010	synonymous variant, V1101V	Development of central nervous system.
rs139608664	*INHA*	0.018	5.716 (1.110 - 36.859)	0.003	synonymous variant, S225R	Peptide hormone metabolism, apoptosis, immune response. TGF-beta superfamily member.
rs4556933	*ACVR1C*	0.019	0.847 (0.735 - 0.976)	0.323	synonymous variant, F38F	Signalling in e.g. embryogenesis and tissue homeostasis. TGF-beta superfamily member.

Candidate gene	Gene name	P-value	OR (95% confidence interval)	MAF in total sample	Consequence (distance from exon, base pairs)	Primary pathway, gene function
rs41302834	*GPR98*	0.022	3.451 (1.031 - 11.557)	0.007	missense variant, D1944N	Development of central nervous system.
rs3736061	*FLT4*	0.023	0.803 (0.665 - 0.973)	0.135	synonymous variant, L252L	Receptor for VEGFC and VEGFD. Lympahgiogenesis.
rs3741849	*PZP*	0.039	1.309 (1.004 – 1.696)	0.068	synonymous variant, K563K, splice region variant	Proteinase inhibitor, highly expressed in late pregnancy serum.
rs34307240	*LCT*	0.026	2.201 (1.038 - 4.515)	0.010	missense variant, D106E	Hydrolase activity and lactase activity, galactose metabolism.
rs36032184	*INHA*	0.027	1.906 (1.058 - 3.346)	0.017	synonymous variant, G109G	Peptide hormone metabolism, apoptosis, immune response. TGF-beta superfamily member.
rs2228048	*TGFBR2*	0.029	0.672 (0.459 - 0.963)	0.030	synonymous variant, N354N	A member of the Ser/Thr protein kinase family and the TGFB receptor subfamily. Cell proliferation.
rs138819536	*INHBA-AS1*	0.032	4.311 (0.926 - 21.758)	0.005	missense variant, R229Q	INHBA Antisense RNA 1
rs1466360	*ADAM12*	0.033	1.152 (1.010 - 1.315)	0.414	intron variant (+30)	A disintegrin and metalloprotease domain-family, actin dynamics signalling pathway, fertilisation

Candidate gene	Gene name	P-value	OR (95% confidence interval)	MAF in total sample	Consequence (distance from exon, base pairs)	Primary pathway, gene function
rs56133834	*TEK*	0.034	3.450 (0.921 - 12.934)	0.004	synonymous variant, E986E	Angiopoietin-1 receptor, embryonic vascular development, angiogenesis.
rs140593977	*TREX1*	0.034	3.448 (0.920 - 12.928)	0.004	downstream gene variant (-44)	3' exonuclease activity. DNA repair. Immune system.
rs1466361	*ADAM12*	0.034	1.152 (1.010 - 1.316)	0.414	intron variant (-46)	A disintegrin and metalloprotease domain-family, actin dynamics signalling pathway, fertilisation
rs148671842	*EHD3*	0.035	2.372 (0.992 - 5.459)	0.007	synonymous variant, E188E	Megakaryocyte development and platelet production. Cholesterol and sphingolipids transport/recycling.
rs115734907	*KDR*	0.035	0.695 (0.483 - 0.979)	0.029	intron variant (+12)	Angiogenesis. Main mediator of VEGF-induced endothelial proliferation, survival, migration, tubular morphogenesis and sprouting.
rs1554286	*IL10*	0.037	1.203 (1.009 - 1.438)	0.153	intron variant (+18)	T-cell growth inhibitory factor. B-cell function enhancer. Allograft rejection.

Candidate gene	Gene name	P-value	OR (95% confidence interval)	MAF in total sample	Consequence (distance from exon, base pairs)	Primary pathway, gene function
rs2453040	*NOTCH2*	0.037	0.818 (0.676 - 0.992)	0.141	intron variant (-45)	Cell-cell signalling. Angiogenesis.
rs116951780	*LCT*	0.038	10.333 (0.829 - 541.218)	0.001	synonymous variant, NMD transcript variant, A921A	Hydrolase activity and lactase activity, galactose metabolism.
rs138894008	*TEK*	0.038	10.333 (0.829 - 541.218)	0.002	missense variant, R479H	Angiogenesis. Signalling in embryonic vascular development.
rs200071734	*FLT4*	0.040	10.114 (0.811 - 529.805)	0.002	missense variant, V157M	Receptor for VEGFC and VEGFD. Lympahgiogenesis.
rs150123876	*ANGPT4*	0.042	0.348 (0.090 - 0.968)	0.007	missense variant, R25H	Angiogenesis. Cell surface interactions at the vascular wall.
rs368518386	*FLT4*	0.046	5.581 (0.799 - 61.819)	0.003	intron variant (-43)	Receptor for VEGFC and VEGFD. Lympahgiogenesis.
rs3736062	*FLT4*	0.046	1.547 (0.988 - 2.374)	0.024	synonymous variant, Y531Y	Receptor for VEGFC and VEGFD. Lympahgiogenesis.
rs7830	*NOS3*	0.049	0.877 (0.769 - 1.002)	0.434	intron variant (+11)	Angiogenesis. Produces NO, vascular smooth cell relaxation, mediates angiogenesis in coronary vessels and activates platelets.

Nitric oxide synthase 3 (NOS3) synthesises nitric oxide (NO), which is a reactive free radical that has a role as a biologic mediator in several processes, including antimicrobial and anti-tumoural activities and neurotransmission. Association of NOS3 has been investigated in several studies and a haplotype including rs7830 was found to protect women from pregnancy hypertension and pre-eclampsia (Muniz *et al.* 2012). However, the association has not been confirmed in meta-analyses (Staines-Urias *et al.* 2012, Buurma *et al.* 2013). Indeed, Staines-Urias et al. found indication that reported associations of NOS3 and other genes to pre-eclampsia might be due to reporting bias, where results are reported selectively (Staines-Urias *et al.* 2012). Other variants in NOS3 are associated with susceptibility to coronary spasm (Chang *et al.* 2003). Alternative splicing and alternative promoters result in multiple transcript variants of NOS3. It is possible that similarly to *FLT1*, only some of the isoforms of NOS3 are relevant in the context of pre-eclampsia thereby making result comparison between studies and data interpretation challenging when information about individual SNP position and studied isoforms is not reported.

Interleukin 10 is an anti-inflammatory cytokine that is most often linked to inflammation suppressing Th2 type immunity (Spellberg & Edwards 2001). The role of interleukin 10 (L-10 in pre-eclampsia has been demonstrated by a murine model of IL-10 deficit mice (Kalkunte *et al.* 2010). We found rs1554286 to associate to pre-eclampsia ($p=0.037$). This SNP belongs to an intronic haplotype, which strongly predisposes women of Bahraini Arab population to idiopathic recurrent miscarriages (Qaddourah *et al.* 2014). The association results from Study IV support the hypothesis that recurrent miscarriages and pre-eclampsia may share common immunological aetiology (Redman & Sargent 2010b, Robertson *et al.* 2003).

The results discussed here concern non-adjusted significance testing from a targeted exomic sequencing. While some of the results may be false positives, the results corroborate several known associations between adverse pregnancy outcomes and candidate genes. Furthermore, the statistical framework of the targeted exome sequencing supports validity of the results: the tail of the p-value distribution of benign variants was as expected (0 results with $p<0.001$ (expected ≤ 1) and 2 results with $p<0.01$ (expected ≤ 4.2)). Concerning the 201 putatively functional variants, one had $p<0.001$ (alpha=0.05) compared to the 0/421 observed among benign variants. Statistical confounders such as stratification would be indicated by inflated p-values, which were not observed in this study. Further studies into regulatory mechanisms and haploblock structures within these genes may reveal the true

functional association underlying these individual preliminary associations.

5.8 Limitations of the study

In Study I, foetal and maternal components of the complement system were not in all cases distinguishable, and foetal genotypes of *C4* genes were studied alongside the maternal genes in this small group of patients. The laboratory methods of this study were completed before most of the foetal DNA of the FINNPEC cohort became available. Therefore, the other genetic association studies were concerned with only the maternal genotype. It is obvious that especially in immunological studies the compatibility of maternal and foetal genotypes and functional pathways is important. The same is true for studies of sFlt1, which is known to be largely of placental origin during pregnancy.

A core problem in placental studies is the fact that the complications underlying defective placentation most likely take place in the early second or even first trimester during the two phases of trophoblast invasion. In study of the post-partum placenta the data is always limited to the condition and structure of the placenta after the processes of later pregnancy and birth. Therefore, conclusions concerning disease aetiology drawn from this material must be conservatively interpreted. However, pathophysiological study of the immunological components in the placenta may serve as a fingerprint of processes that led to the disease development. At the very least, they will describe the immunological stress the placenta was subjected to at the time of birth.

Candidate gene studies are often balanced on rigorousness of data and sample size and the labour and finances available. These problems can to a degree be overcome by next generation sequencing methodology, such as the targeted exome sequencing in Study IV. However, our sequencing gene studies with limited sample sizes have shown that sequencing has benefits such as coverage of the intronic, splicing, and regulatory regions. When sequencing protocols can be adjusted individually for each amplicon, even technically challenging genomic regions can be analysed. While statistically limited, the data from sequencing studies is comprehensive and allows for a holistic approach to data analysis including exploration of the haploblock structures spanning across several exomes.

This study is concerned with candidate genes in pre-eclampsia and different aspects of humoral innate immunity. Immunological processes function systemically and locally in an intertwined web of interactions between humoral and cellular immunity as well as innate and adaptive immunity. While humoral innate immunity and complement system is the first line of defence with specific role of self-nonself detection, I have not explored possible cellular immunological contributors or down-stream immunological processes within adaptive immunity that may also have an important role in aetiology of pre-eclampsia.

This study was done in Finnish patients and controls. Pre-eclampsia is a complex disease whose aetiology may vary between populations. Finland has a unique population history, and consequently, particularly non-common loss-of-function alleles are overrepresented in the Finnish population (Lim *et al.* 2014). It is beneficial if several populations are studied in pursuit of global factors of complex diseases.

This study did not explore functional consequences of the genetic findings. Mechanistic validation would add weight to the associations described in this study.

5.9 Future perspectives

5.9.1 Future of pre-eclampsia research

The results of this study give rise to several important research questions. Replication of the protective variants in *FLT1* is of particular interest in other populations but also in Finnish samples of children born from pre-eclamptic pregnancies. The structure and genomic context of *FLT1* is poorly understood and warrants further research.

Candidate genes within the complement system should be further studied, as only two genes of the complement system were sequenced as part of this study. Likewise, the *C4* genotyping in different disease sub-phenotypes is an important next step.

Replication of our results in ethnically diverse sample cohorts will show, if the associations found are unique to Finnish population. In either case, detailed research of the candidate genes with associating variants may eventually pinpoint the associating element and function of the candidate

proteins in pathophysiology of pre-eclampsia.

Functional studies to explore the underlying mechanism of genetic association are required to validate the genetic associations found here.

5.9.2 Therapeutic suggestions

The role of sFlt1 in the aetiology of pre-eclampsia remains enigmatic. However, it is plausible from this and other studies, that sFlt1 plays an important role in at least aggravation of pre-eclampsia. Removal of sFlt1 out of the maternal serum could be one possible way of treatment to restore angiogenic balance in pre-eclamptic patients (Schrey-Petersen & Stepan 2017). Neutralising antibodies may also be explored to prevent the effects of excess sFlt1 in the maternal circulation.

Eculizumab was one of the first drug that inhibits complement activation and was used to treat a pre-eclamptic patient with HELLP with success (Burwick & Feinberg 2013). Eculizumab targets the terminal pathway of complement activation. While supporting the effect of complement activation in pre-eclampsia pathogenesis, the results of this study did not address the effect terminal pathway of complement activation specifically and therefore recommendations concerning use of Eculizumab in treatment of pre-eclampsia warrants further studies.

The results of this study suggest, that with further research, it may be possible to identify a more specific upstream target within the complement system that may, in suitable patients, be influenced with therapeutics. This would prevent the release of anaphylatoxins which are released as complement cascade advances and thereby limit the inflammatory response effectively.

Increasing knowledge of the particular traits enriched in the Finnish population may also open avenues for development of personalised risk evaluation and tailored intervention strategies (Kaariainen *et al.* 2017).

6. CONCLUSION

Pre-eclampsia is a complex disease which is reflected in its immunogenetic foundation. Immunological health of the pregnancy is constituted by a network of interacting immunological processes of maternal and foetal origins (Parham & Moffett 2013). Identifying the appropriate pathways in targeting treatment for any pre-eclamptic woman requires more studies of the immunogenetic framework while keeping in mind the evolutionary consequences of the findings. The human pregnancy and birth can be seen as a life-history bottleneck (Brown *et al.* 2013). The study of the maternal and foetal implications of reproductive complications opens a unique opportunity to broaden our understanding of the human evolutionary process (Brown *et al.* 2013).

This study adds to the growing body of evidence, which indicates that processes within the innate immunity play a central role in development of pre-eclampsia. Furthermore, our findings support the hypothesis that recurrent miscarriages and pre-eclampsia may share common immunological aetiology (Redman & Sargent 2010b, Robertson *et al.* 2003). The immunological connection between pre-eclampsia and miscarriage warrants further study by replication of our results in suitable cohorts of consisting of patients with reproductive failure.

The results in of this study highlight the importance of careful sub-phenotyping in pre-eclampsia research. It is likely that the patient pool is comprised of individuals with varying aetiological determinants, but who present with similar symptoms of proteinuria and hypertension in the second half of the pregnancy. Therefore, careful phenotyping should be at the core of research studies on pre-eclampsia. When comparing results to previous studies, it is essential that the interpretation is adjusted to the phenotype of the studies in question. Moreover, in any future study designs standardised data collection should be at the core of data collection. It is of paramount importance that studies be compared and replicated in well-characterised and compatible patient samples.

Pregnancy brings about tremendous changes in the body fluid volume and distribution. Fundamentally, pre-eclampsia is a disease that reflects an underlying failure in regulating vascular and hemodynamic changes in pregnancy, which may be due to dysregulation of immunological processes and the consequential loss of tolerance to the allogenic foeto-placental unit (Zhou & Wu 2013).

The work presented here underlines the great potential of genetic studies in prediction, diagnosis, and treatment of pre-eclampsia. In the field of complex disease genetics, the recent technological advances allowing for more cost-effective and detailed analysis of the genome hold great promise. However, hypothesis based candidate studies, when undertaken using a carefully characterised and representative pool of patients, still have the potential to produce important advances towards a better understanding of the enigma that is pre-eclampsia. The results of this study may open novel avenues for treatment development in pre-eclampsia as well as tools for identifying the pool of patients that is most likely to benefit from intervention to promote health in later life.

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9. INTERNET LINKS

[a] FINRISK study description: https://www.thl.fi/en/web/thlfi-en/research-and-expertwork/population-studies/the-national-finrisk-study.

[b] FINRISK ethical considersations and detailed study description: https://www.thl.fi/documents/10531/1921702/2015+FINRISK+description_for_researchers_final.pdf/fc952cba-86f6-4ef5-8ef2-fa13c23173c3

[c] Loss of function tool: https://github.com/ensembl-variation/VEP_plugins/blob/master/LoFtool.pm

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Complement activation and regulation in preeclamptic placenta

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Preeclampsia (PE) is a common disorder of pregnancy originating in the placenta. We examined whether excessive activation or poor regulation of the complement system at the maternal–fetal interface could contribute to the development of PE. Location and occurrence of complement components and regulators in placentae were analyzed. Cryostat sections of placentae were processed from 7 early-onset PE (diagnosis <34 weeks of gestation), 5 late-onset PE, 10 control pregnancies, and immunostained for 6 complement activators and 6 inhibitors. Fluorescence was quantified and compared between PE and control placentae. Gene copy numbers of complement components *C4A* and *C4B* were assessed by a quantitative PCR method. Maternal *C4* deficiencies (≥1 missing or non-functional *C4*) were most common in the early-onset PE group (71%), and more frequent in late-onset PE compared to healthy controls (60 vs. 38%). Complement C1q deposition differed significantly between control and patient groups: controls and early-onset PE patients had more C1q than late-onset PE patients (mean $p = 0.01$ and $p = 0.005$, respectively). C3 activation was analyzed by staining for C3b/iC3b and C3d. C3d was mostly specific to the basal syncytium and C3b/iC3b diffuse in other structures, but there were no clear differences between the study groups. Activated C4 and membrane-bound regulators CD55, CD46, and CD59 were observed abundantly in the syncytiotrophoblast. Syncytial knots, structures enriched in PE, stained specifically for the classical pathway inhibitor C4bp, whereas the key regulator alternative pathway, factor H (FH) showed a wider distribution in the placenta. Differences in C1q deposition between late- and early-onset PE groups may be indicative of the different etiology of PE symptoms in these patients. Irregular distribution of the complement regulators C4bp and FH in the PE placenta and a higher frequency of *C4A* deficiencies suggest a disturbed balance between complement activation and regulation in PE.

Keywords: preeclampsia, complement, pregnancy, placenta, immunohistochemistry, innate immunity

INTRODUCTION

Preeclampsia (PE) is a serious complication of human pregnancy. It can lead to multi-organ dysfunction and, rarely, to a life-threatening convulsive condition, eclampsia (1). PE affects 3–5% of pregnancies in all ethnic groups. The development and progression of the disease are unpredictable. Presently, delivery of the placenta remains the only cure for PE. The etiology of PE is still largely unknown. Because of the unique challenge that the fetoplacental unit poses to the maternal immune system PE could involve a non-classical-type incompatibility (2).

The complement (C) system is a phylogenetically ancient means of self–non-self discrimination. It plays a central role in innate immune defense, clearance, and as a mediator of the adaptive immunity (3). Complement activation is regulated by soluble and membrane-bound inhibitor molecules. Activation of the C system releases potent anaphylatoxins, which generate inflammation by mediating chemotaxis, increased vascular permeability, smooth muscle contraction, and leukocyte activation. In addition, C has been indicated in the homeostatic clearance of waste products as well as in regenerative processes. The discrimination potential of C goes beyond that between self and non-self, because C can distinguish non-viable tissue components and cells from viable ones. In particular, a disturbed binding of the soluble regulator factor H (FH) to injured or altered host cells could allow an alternative pathway-mediated attack against such target. Also, exposed subcellular or disturbed membrane structures could initiate both classical and alternative pathway activation, either directly or via activating molecules like C-reactive protein (CRP) or natural antibodies.

Disturbances in C activity can predispose to infections or to a systemic lupus erythematosus (SLE)-like immunoinflammatory syndrome. The latter has been related to an inadequate waste

disposal function of the classical pathway (4). These types of syndromes tend to become worse during pregnancy, possibly because of a greater challenge to the clearance system posed by material derived from the placenta. On the other hand, disturbances in C regulation can lead to such catastrophic consequences as the atypical hemolytic uremic syndrome (aHUS) and other forms of thrombotic microangiopathy (TMA) (5). Central to these is C attack against endogenous tissue structures, endothelial cells, and blood cells that can lead to vascular damage and organ failure, notably in kidneys. Pregnancy is a well-known potential trigger for such syndromes.

The depth of placentation required for a healthy human pregnancy presents a unique challenge to regulation of the maternal immunological processes (6, 7). For the trophoblast invasion and healthy placentation to occur, the fetal cells must avoid recognition by and activation of the C system (7). A risk for the latter exists because of the potential development of maternal antibodies or spontaneous activation of the C system by exposed villous structures, which are often observed in the PE placentae by microscopy.

A well regulated C system is a prerequisite for a healthy pregnancy (8, 9). Recently, mutations in C regulatory genes have been reported in women with recurrent pregnancy loss (10). Recently, a case report was published, where treatment with the C5 inhibitor eculizumab prolonged HELLP (hemolysis, elevated liver enzymes, and low platelets)/PE pregnancy by 17 days. The treatment resulted in a clinical improvement of the patient and normalization of her lab parameters supporting the role of C in the pathogenesis of PE (11). Up to our knowledge, however, the C system has never been described in its entirety in the PE placentae in comparison with healthy control placentae. In particular, little information is available on the possible role or dysfunction of the two major soluble inhibitors FH and C4b binding protein (C4bp) in the placenta during pregnancy.

Two forms of *C4* exist, *C4A* and *C4B*, which are the targets for control by the C4bp regulator protein. Genes for *C4A*, *C4B*, as well as for C2 and factor B, are encoded in the MHC class III region in the human chromosome 6p21.3. *C4* genes are polymorphic, variations in gene copy numbers exist and deficiencies are common (12). Deficiencies in C2 and *C4A* predispose to SLE, a disorder known to worsen during pregnancy (13, 14). Because of the vascular disturbance in PE and its similarity to many diseases involving C dysfunction (SLE, phospholipid antibody syndrome, aHUS) we found it reasonable to hypothesize that an imbalance between C activation and regulation could be involved in PE (**Figure 1**).

To test the involvement of C in PE, we have analyzed immunohistochemically the deposition and expression of key activating components and regulators of the C system in preeclamptic placentae in relation to disease onset and in comparison to healthy placentae. The results favor the hypothesis that an insufficient complement function is linked to an inability to clear away trophoblast material from the placenta. As a consequence, the material deposits in fibrinoid clusters and could cause an endothelial–vascular disorder in the maternal circulation.

MATERIALS AND METHODS

PATIENTS

For this study, we chose randomly 12 women with PE and 10 controls without PE (**Table 1**) from the prospective arm of the Finnish Genetics of Preeclampsia Consortium (FINNPEC) cohort. While FINNPEC is a multicenter study, all women in this study

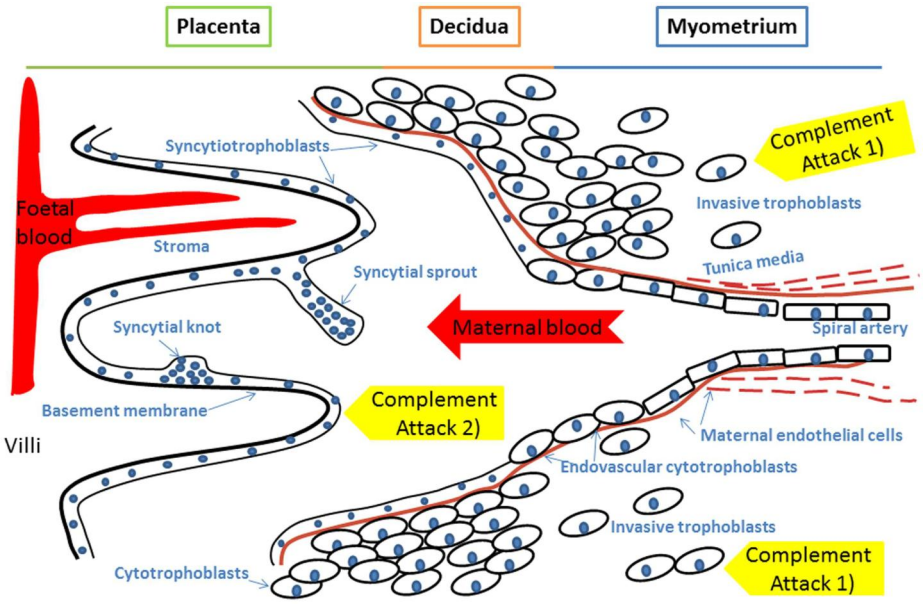

FIGURE 1 | A model of innate immunity incompatibility between maternal and fetal cells in preeclampsia and the maternal immune system. Failure of complement regulation on fetal tissue or excessive activation of the maternal complement system could result in complement attack against 1) invading trophoblast cells or 2) placental syncytiotrophoblast that represent the discordant interfaces. Accordingly, an imbalance between complement activation and regulation could contribute to the pathogenesis of preeclampsia. Specific foci for complement to attach could include syncytial bodies (apoptotic syncytial knots and syncytial sprouts), which are observed more often in preeclamptic placentae than in healthy controls.

Table 1 | Clinical characteristics of the study population.

	Controls $N=10$	Late-onset PE, $n=5$	Early-onset PE, $n=7$
Age	30.6 ± 3.1	33.8 ± 4.1	31 ± 6.5
Gravidity	1.8 ± 0.8	1.2 ± 0.5	1.9 ± 1.1
Parity	0.6 ± 0.8	0.2 ± 0.5	0.6 ± 1.0
Maternal BMI (kg/m^2)	22.9 ± 2.7	21.0 ± 2.2	22.3 ± 2.4
Hypertension before pregnancy	1/10	1/5	2/7
Celiac disease	0/10	1/5	0/0
Thrombophilia	0/10	0/5	1/7
PE in previous pregnancy	0/10	1/5	1/7
Early pregnancy systolic BP (mmHg)	114 ± 8	117 ± 7	129 ± 10[b,c]
Early pregnancy diastolic BP (mmHg)	73 ± 8	77 ± 5	82 ± 9[a]
Highest systolic BP (mmHg)	128 ± 13	166 ± 10[b]	167 ± 16[b]
Highest diastolic BP (mmHg)	87 ± 10	109 ± 8[b]	118 ± 9[b]
Highest proteinuria (g/24 h)	–	1.8 ± 0.4	5.7 ± 3.8[b,c]
Gestational weeks at birth	40 ± 2	38 ± 2[a]	33 ± 4[b,c]
Birth weight (g)	3646 ± 282	2938 ± 423[b]	1842 ± 544[b,d]
Complications			
IUGR	–	–	3/7
Placental insufficiency	–	–	2/7
HELLP	–	1/5	1/5

[a] *Significant in <0.05 level when compared with controls.*
[b] *Significant in <0.01 level when compared with controls.*
[c] *Significant in <0.05 level when compared with late-onset group.*
[d] *Significant in <0.01 level when compared with late-onset group.*
Mean ± SD values are shown.
PE, preeclampsia; BMI, body mass index; BP, blood pressure; IUGR, intrauterine growth restriction; HELLP, hemolysis; elevated liver enzymes, low platelets.

delivered at the Helsinki University Central Hospital. Placental samples (nine-site biopsies) were collected after delivery from the patients. All pregnancies were singletons and exclusion criteria were multiple pregnancies or maternal age <18 years. An additional exclusion criterion was a known autoimmune disease such as SLE. All subjects provided a written informed consent and the FINNPEC study protocol was approved by the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa.

Preeclampsia was defined as hypertension and new-onset proteinuria occurring after 20 weeks of gestation. Hypertension was defined as systolic blood pressure of 140 mmHg or more, and/or a diastolic blood pressure of 90 mmHg or more after 20 weeks of gestation. Proteinuria was defined as the urinary excretion of ≥0.3 g protein in a 24-h specimen, or 0.3 g/l or, in the absence of concurrent quantitative measurement, at least a "2+" or more, or two "1+" proteinuria dipstick readings with no evidence of urinary tract infection. PE was considered severe if blood pressure was ≥160/110 mmHg, or proteinuria exceeded 5 g/24 h, or symptoms like cerebral or visual disturbances or abdominal pain appeared. Intrauterine growth restriction (IUGR)/placental insufficiency was defined as birth weight below −2SD and/or umbilical artery resistance ≥+2SD according to gestational age specific standards (15) without known etiology unrelated to the aims of the present project (e.g., congenital malformation syndromes and chromosomal defects). We have divided the PE women into two groups according to the weeks of gestation at diagnosis: early-onset <34 weeks of gestation ($n=7$), late-onset ≥34 weeks of gestation ($n=5$).

Placental samples were chosen preferentially from patients with severe and early-onset PE. Chronic hypertension (an elevated blood pressure that predated the pregnancy or detected before mid-pregnancy) was observed in three PE women and one woman in the control group (**Table 1**). Two women had HELLP syndrome. One woman of the late-onset PE group had celiac disease and one patient of the early-onset PE group had thrombophilia caused by mutation in the coagulation factor FII.

SAMPLE PREPARATION

Approximately 1 cm wide tissue samples from the placentae were dissected using a scalpel and scissors and placed in a cryotube for preservation. Following the nine-site procedure, the placenta was visually divided into nine pre-specified regions and one sample was taken from each region. Within 2 h of the delivery of the placenta, the cryotubes containing samples were placed into the inner compartment of a nested metal holder. Approximately 150 ml of liquid nitrogen was added to the outer compartment to cool down the 150 ml isopropanol poured into the inner compartment. According to a standardized tissue-preserving collection procedure, the samples were left for 20 min to freeze slowly through the isopropanol pool. When isopropanol reached a floury frozen state, the cryotubes were stored at −80°C. For our study, one region (no. 5) was immunohistochemically analyzed from all samples.

ANTIBODIES AGAINST COMPLEMENT FACTORS

The antibodies used are listed in **Table 2**. Primary antibodies were chosen to detect either the activating components or regulators of the alternative and classical pathways of complement activation. Soluble endoglin (s-eng) was used as a positive control for changes in PE placenta (16).

Anti-C3c antibody was used to detect C3b and iC3b, which are the products of alternative pathway activation and amplification and of subsequent C3b inactivation. C3d fragment was separately stained for because the C3d antibody recognizes the C3dg fragment, which remains surface bound after the release of C3c. FH binds to the C3b molecule on the self-cell surface, where it can be detected by the FH antibody. The C1q antibody recognizes several different structures of the classical pathway activating C1q molecule. The C4c antibody recognizes the native C4 molecule

Table 2 | Primary antibodies used for immunofluorescence stainings.

Antibody	Type	Dilution	Source[a]	Role
C1q	Rabbit pAb	1:1000	DAKO	CP component
C4c	Rabbit pAb	1:400	DAKO	CP component
C4bp	Sheep pAb	1:200	The Binding Site	CP regulator
CRP	Mouse mAb	1 μg/ml	Fitzgerald	CP activator
C3c	Rabbit pAb	1:1000	DAKO	AP component
C3d	Rabbit pAb	1:1000	DAKO	AP component
Factor H	Goat pAb	1:400	Calbiochem	AP regulator
C9	Goat pAb	1:400	Quidel	TP component
MCP (CD46)	Mouse mAb	1 μg/ml	IBGRL	AP and CP regulator
Bric 230 (CD55)	Mouse mAb	1 μg/ml	IBGRL	AP and CP regulator
Bric 229 (CD59)	Mouse mAb	1:200	IBGRL	TP regulator
CR1	Mouse mAb	1 μg/ml	AbD Serotec	AP and CP regulator
s-Endoglin	Mouse mAb	2 μg/ml	Santa Cruz	PE indicator

[a] *DAKO, Glostrup, Denmark; The Binding Site, Birmingham, UK; Fitzgerald Industries International, North Acton, MA, USA; Calbiochem, Merck KGaA, Darmstadt, Germany; Quidel Corporation, San Diego, CA, USA; IBGRL The International Blood Group Reference Laboratory, Bristol, UK; AbD Serotec, Oxford, UK; Santa Cruz Biotechnology, Inc., Dallas, TX, USA.*

pAb, polyclonal antibody; mAb, monoclonal antibody.

CP, classical pathway; AP, alternative pathway, TP, terminal pathway; PE, preeclampsia.

(both C4A and C4B) as well as the activation product C4b and its inactivated form iC4b. For detection of C4bp, a cofactor for C4b in activation, a polyclonal sheep antibody was used. Membrane-bound DAF (CD55) and MCP (CD46) were analyzed by specific mouse monoclonal antibodies.

IMMUNOFLUORESCENCE STAINING

The frozen tissue samples were cryosectioned at 5 μm and when possible two or three serial sections were laid per each slide. The dried sections were rinsed with phosphate-buffered saline (PBS) and moist samples were blocked against non-specific binding with 1% bovine serum albumin (BSA) in PBS for 15 min in a humid chamber. Excess liquid was discarded and the first antibody was pipetted to the sample in 1% BSA/PBS as detailed in **Table 2**. One section on the slide was used for a mock staining by treating it with only 1% BSA/PBS without the first antibody (**Figures 4D,H,L**; **5D,H** and **6D,H,L**). After washing for five times (1 min each) the second antibody was pipetted at a 1:300 dilution in 1% BSA/PBS. Second antibodies used were Alexa 488-labeled antibodies against goat, rabbit, mouse, and sheep immunoglobulins (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). After 20-min incubation and five 1-min rinses, excess liquid was removed and a mounting medium was added. Stained sections were kept at +4°C in the dark until documentation on the same day.

HISTOCHEMISTRY

One slide from each sample was used for standard automated hematoxylin and eosin (HE) staining to ensure diagnostic-level consistency and to obtain a histological reference point for the immunofluorescence (IFL) analyses.

IMAGING AND HISTOLOGICAL ANALYSIS

Data were collected using standardized fluorescence microscopy settings, where all slides were photographed with 10×, 20×, and 40× magnifying objectives. Exposure times per each magnification were 20, 50, and 83.3 ms. Images were collected using Olympus DP Manager (ver. 2.2.1.195) and Olympus DP Controller (ver. 2.2.1.227) image capture softwares with Olympus BX51 fluorescence microscope camera.

The same protocol and machinery for imaging was used for histochemistry preparations. Images captured from HE stained samples were used to identify key structures and to characterize the placenta. The structures to be identified included stem villi, small villi, villous fibrinoid (i.e., as a part of a villus, often replacing syncytium), fibrinoid necrosis, syncytiotrophoblast (STB), cytotrophoblast, fetal arterial endothelium, and syncytial bodies (incl. syncytial sprouts and syncytial knots). The occurrence of syncytial bodies and fibrinoid structures was semiquantified by grading (0 = none observed, 1 = counted 1–3, 2 = >3, 3 = all over, cannot be counted). Structural integrity was measured by intactness of syncytium and special attention was paid on shedding of syncytial cells. Signs of nuclei of apoptotic cells were looked for and noted.

GENETIC ANALYSIS

To correlate the C4 deposition observed in the placentae with the functional *C4* genes, *C4A* and *C4B* gene copy numbers and a silencing *C4A* mutation were analyzed using a previously published protocol (12). Briefly, a SYBR® Green labeled real-time quantitative polymerase chain reaction (qPCR) with a specified concentration range approach was used to obtain numbers of *C4* and to detect deficiencies due to CTins, which renders the affected *C4A* non-functional. Two copies of *C4A* and *C4B* are considered the normal genotype and while deviations from the four-gene norm are common, individuals with less than two genes for either *C4* gene or individuals with *C4A* CTins mutation are considered *C4* deficient. DNA for the qPCR protocol was extracted from whole blood samples of mothers and from umbilical cord blood samples collected post-partum from the placenta.

Blood samples were stored in −80°C and DNA was later extracted using Macherey-Nagel NucleoSpin Blood XL kit (Macherey-Nagel GmbH & Co., KG Düren, Germany). Extracted DNA was stored at −80°C until used in the analysis.

STATISTICAL ANALYSIS

ImageJ 1.46 and Fiji-win32 softwares were used to quantify the intensity of fluorescence in the fixed magnification images. These were chosen to minimize the variation of staining quality and tissue quality between individuals, which was more apparent at the highest levels of magnification. To correct for false positive readings resulting from background autofluorescence, mean intensity +1 SD ($\bar{X}+\sigma$) was determined to be 7 at 20 ms exposure and 15 at 50 ms exposure. This was calculated from analysis of negative controls (**Figures 4D,H,L**; **5D,H** and **6D,H,L**). Using the appropriate zero thresholds each image was analyzed for several parameters of fluorescence intensity. Sum was defined as mean intensity * area of positive fluorescence in pixels ($\bar{X} \times \sigma$). The purpose of using different parameters was to differentiate between different patterns as well as intensity of fluorescence. Log_{10} transformation was used to normalize the image capture data and normality of the transformed data was verified by Shapiro–Wilk W test (data not shown). An independent-samples t-test for analyzing the significance of differences between means of values obtained from patient groups and controls was carried out for key statistical parameters including sum and mean as well as the clinical measurements. For the high-intensity analysis, the top 75% proportion of fluorescence histogram was determined for each image using ImageJ 1.46 software and the calculated maximum fluorescence value (**Figure 2**). A filter was placed at the calculated 75% minimum value creating representative images of high-intensity regions. Statistical testing of the high-intensity area percentage was done as above (data not shown). Furthermore, Pearson-correlation of high-intensity proportions between different stainings was calculated for each patient group independently. Positive correlations were used as an indicator of two components observed in the same sample, while negative correlations were interpreted as two components occurring in different samples. Fisher's exact test was used to analyze for differences between segregation of *C4* gene deficiencies between groups of patients, and independent-samples t-test was used to assess the association of *C4* gene deficiencies and immunohistochemistry fluorescence sum and mean values.

RESULTS

Activating components and regulators of the C system as well as s-endoglin were found to be deposited in the placenta in a structure-specific manner showing differences between patient groups and controls. In the following, the results are presented according to C pathways (**Figure 3**).

CLASSICAL PATHWAY

C1q

C1q was observed at the STB layer in 5/10 controls but in none of the early- onset PE patients and only in one of the late-onset

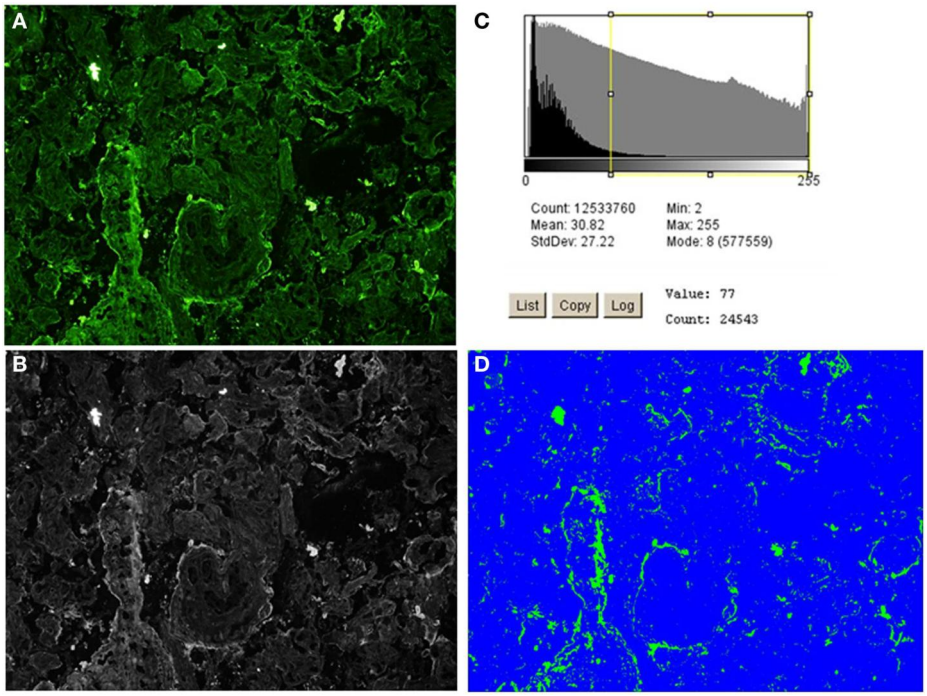

FIGURE 2 | High-intensity analysis workflow of C4bp staining of an early-onset preeclamptic placenta using ImageJ 1.46 software. The image is processed through steps **(A–D)** to produce a quantification of the high-intensity fluorescence areas, which correspond to the structures where **(C)** deposition/expression is most conspicuous. **(A)** The original image. **(B)** Black and white rendering of the image in **(A)**. **(C)** Threshold set at 75% positive fluorescence (calculated to be value 77 for this image). **(D)** Area (in pixels) of positive signal (in green), % area is given in output and compared across and between the patient groups.

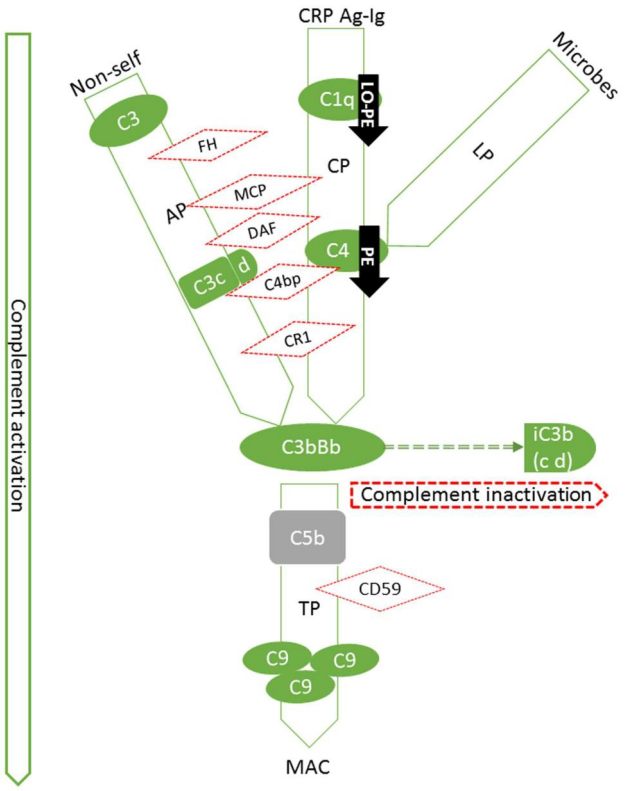

FIGURE 3 | Summary of expression patterns of complement components in the placenta. Our findings are highlighted in black arrows (PE: preeclampsia, LO-PE: late-onset preeclampsia). Pictured are the components that we studied in their respective position in the activation cascade (green solid lines and shapes) or regulatory network (red dotted lines and shapes). The C system is composed of approximately 20 plasma proteins, which can be activated in a stepwise cascade via the classical (CP, via Ag-Ig: immunocomplexes or CRP: C-reactive protein), lectin (LP), or the alternative pathway (AP). In addition, there are approximately 15 components that act as receptors or protective molecules on cell membranes. All three pathways result in the activation of the main complement component C3 and thereafter of the terminal pathway causing the formation of membrane attack complexes (MACs) and ultimately target cell damage. C5b (not studied, in gray) is the activated component at the onset of terminal pathway. FH is a regulator of the alternative pathway, where its main role is to act as a cofactor for Factor I in the cleavage of C3b into iC3b. Similarly in the classical pathway, cleavage of C4 to activated form C4b is inhibited by a potent regulator C4bp. C3b and C4b are both generated by activation of not only the classical, but of the lectin pathway as well. C1q is a potent activator of the classical pathway. Binding of the complex ($C1qr_2s_2$) activates the C4 step. Together with decay accelerating factor (DAF; CD55) and membrane cofactor protein (MCP, CD46), C4bp regulates the progression of the classical C pathway by controlling the formation and function of the classical pathway C3 convertase, C4b2a. Like FH, MCP can also act as a cofactor in C3b inactivation. In the classical pathway, DAF accelerates the disassembly of C4b2a and in the alternative pathway that of C3bBb. DAF is a glycosylphosphatidylinositol-anchored membrane molecule. Complement receptor type 1(CR1, CD35) is a membrane-bound regulator expressed primarily by bone-marrow derived cells.

PE patients. Overall, we found less C1q in the PE patients when compared with normal controls (**Figures 4A–C**). In about half of the cases, C1q was observed in the endothelia of placental vessels. In the ImageJ analysis, the amount of C1q deposition was higher in controls and early-onset PE group than in late-onset PE group (mean $p = 0.01$ and mean $p = 0.005$, respectively) (**Tables 4** and **5**). Accordingly, the area of high-intensity regions for C1q staining was significantly smaller in the late-onset PE cases than in controls ($p = 0.005$) and in the early-onset PE group ($p = 0.011$). In the areas of high-intensity staining, the tissue structure was breaking down suggesting an on-going necrotic process. C1q was frequently found in the stromal areas of the larger villi. Samples with large villi containing C1q abundantly were observed more frequently in the patients (both groups combined) vs. controls (58 vs. 40%), especially in the early-onset PE group (71%). C1q was present also in the fibrinoid necrotic areas especially in the placentae of patients in the early-onset PE group. Less C1q was seen in the fibrinoid areas of the late-onset group or normal controls.

C4 gene numbers

C4A or *C4B* deficiencies were found almost twice as often in early-onset PE patients than in healthy controls (**Table 3**). *C4A* deficiencies were found in 40% (2/5) of PE mothers with late-onset disease and 43% (3/7) of PE mothers with early-onset disease. None were observed in the controls ($n = 7$). Because of the small number of samples, the difference was on the borderline statistically significant ($p = 0.055$ Fisher's exact two-sided test). Only two individuals had total *C4* deficiency (total *C4A* deficiency in one control mother and total *C4B* deficiency in one child born from early-onset PE pregnancy).

C4

C4 deposits were observed mainly in the STB layer, either in the apical membrane or throughout the syncytium. There was no clear difference between the patient groups and controls in this pattern (**Tables 4** and **5**). A particular staining for C4 was seen in clusters formed from the STB layer. These clusters represent syncytial bodies (i.e., syncytial knots or sprouts) and shedding of the syncytium. The number of C4 clusters was slightly higher in the preeclamptics (**Figures 5E–G**). The intensity of the C4 fluorescence mean or sum values did not associate with *C4A* or *C4B* deficiencies of the mother or fetus.

C4bp

C4bp is an inhibitor of the classical pathway occurring usually physiologically in complex with the anticoagulant protein S. Overall, C4bp was found deposited particularly in small syncytial bodies, which appeared as brightly staining particles attached or sometimes shed from the syncytium (see **Figures 1** and **5B**). Characteristically, these were seen as dense clusters of bright staining, which were interpreted as small necrotic, apoptotic, or fibrinoid tissue fragments, typically syncytial knots (**Figure 5A**). In contrast to other C regulators C4bp was not deposited in a circumferential continuum on the syncytium, while it was typically observed only on the apical surface of the syncytium. Of the controls 80% (8/10) showed apical C4bp staining in the STB whereas in PE cases 42% (5/12) had some C4bp deposition on the STB layer (**Figure 5**). The stromas of the villi were negative and also the placental endothelium was mostly negative for C4bp. In a few

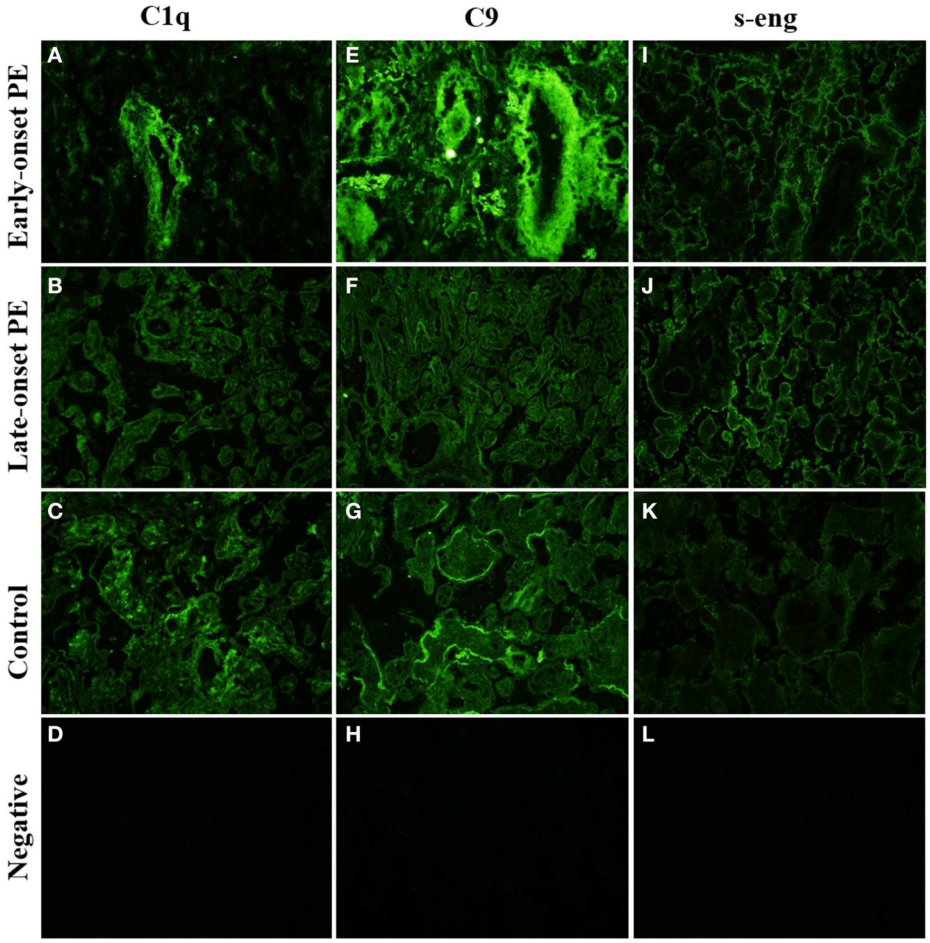

FIGURE 4 | Side-by-side comparison of C1q, C9, and s-Eng staining of the same individual placentas. C1q is absent from large villi of the late-onset PE control. **(B)** There is a negative correlation between high-intensity areas of C1q and C9. s-Endoglin is deposited on the syncytiotrophoblast of PE placentae **(I,J)** and absent from the control placenta **(K)**. Top row **(A,E,I)**: early-onset PE. Middle top row **(B,F,J)**: late-onset PE. Middle bottom row **(C,G,K)**: control. Bottom row **(D,H,L)**: negative control (antibody I omitted). 20× magnification.

Table 3 | Frequency of *C4A* and/or *C4B* deficiencies in preeclamptic (PE) patients and controls.

	C4A or C4B deficiency		C4A deficiency		C4B deficiency	
	Maternal	Fetal	Maternal	Fetal	Maternal	Fetal
PE (pooled) ($n = 12$)	0.667	0.700	0.417	0.300	0.333	0.400
Early-onset PE (7)	0.714	0.667	0.429	0.333	0.286	0.333
Late-onset PE (5)	0.600	0.750	0.400	0.250	0.400	0.500
Control (8)	0.375	0.500	0	0.375	0.375	0.250

placentae small necrotic/fibrin containing areas were intensely stained for C4bp (**Figure 5C**). These were observed in both cases and controls.

C-reactive protein and Complement Receptor type 1 (CR1; CD35) were tested with four samples representing one early-onset PE, two late-onset PE and one control specimens. Both staining were negative. CRP and CD35 were subsequently omitted from the protocol.

ALTERNATIVE PATHWAY

C3

In general, C3 (C3b, iC3b) detected by an antibody against C3c was abundantly present in the placenta. It was found in the STB layer and in the villous stroma (**Figures 6A–C**). In 4 out of 10 control placentae (40%) C3 reactivity was observed on the apical and basal linings of the STB layer. In contrast, this was observed only in one out of seven samples (14%) of the early-onset PE group.

Table 4 | Complement and s-endoglin deposition in early- and late-onset preeclampsia (PE) vs. control.

	Type of PE onset	C3b/iC3b			C1q			C4bp			C4		
		Control			Control			Control			Control		
		t	*p*	df	*t*	*p*	df	*t*	*p*	df	*t*	*p*	df
Sum	Late	1.735	0.106	13	**2.793**	**0.015**	**13**	−0.487	0.635	13	0.969	0.352	12
	Early	−0.671	0.512	15	−0.530	0.604	15	−0.667	0.516	14	0.486	0.635	14
Mean	Late	1.718	0.110	13	**2.993**	**0.01**	**13**	−0.374	0.714	13	0.703	0.495	12
	Early	−0.607	0.553	15	−0.091	0.929	15	−0.507	0.620	14	0.432	0.672	14
	Type of PE onset	**FH**			**C3d**			**C9**			**s-Endoglin**		
		Control			Control			Control			Control		
		t	*p*	df	*t*	*p*	df	*t*	*p*	df	*t*	*p*	df
Sum	Late	0.059	0.954	13	−0.217[a]	0.836	5.463	0.825	0.424	13	*1.842*	*0.090*	*12*
	Early	−0.913[a]	0.378	12.533	−0.365[a]	0.723	9.333	−0.129	0.899	15	1.524	0.150	14
Mean	Late	0.067	0.947	13	0.097	0.924	13	0.722	0.454	13	**2.825**	**0.015**	**12**
	Early	−0.812[a]	0.431	12.927	−0.009	0.993	15	−0.215	0.833	15	*1.815*	*0.091*	*14*

Mean fluorescence intensity and sum of intensity are compared between early- and late-onset PE and controls. C1q and soluble endoglin show significant or borderline significant differences.

[a] *Equal variances not assumed.*

Bold significant to the 0.05 level.

Italics borderline significant.

df, degrees of freedom.

In half of all samples weak staining for C3 was also observed in the placental endothelium, without clear differences between the study groups.

The intensity of C3 deposition appeared weaker in the PE group and even more so in the late-onset group when compared with normal pregnancy (**Figures 6B,C**).

The samples were stained separately also for C3d because it is the C3 activation product that remains covalently bound in areas of C3b deposition thus reflecting a longer period of C activation. C3d deposition was also present in the placenta (**Figures 6E–G**). Reactivity was seen in the villous stroma but a clearer pattern was observed in the syncytium. C3d reactivity was observed mainly in the basal membrane of the syncytium (14/22). The pattern on the syncytial basement membrane was either partially or more fully circumferential lining of the villi (**Figure 6G**). No clear difference was observed between the patient groups and normal controls in the C3d abundance or pattern (**Tables 4** and **6**).

Factor H

Factor H is a key regulator of the C amplification cascade in the alternative pathway. There was significant variation in the FH staining patterns in different placentae (**Figures 6I–K**). In the control placentae, FH localized evenly to the STB layer in 70% (7/10) of the placentae. Often both the apical and the basal layer were stained. Apical side of the STB layer was sometimes more strongly or exclusively stained.

In certain PE placentae, only the STB layer of some villi and fibrin clusters had become strongly stained for FH, while the rest of the tissue remained negative (**Figure 6K**). In other samples, villus stroma was FH-positive throughout, with distinct patterns in the fetal endothelium, STB layer, and in fibrin clusters (**Figure 6I**). The basal–apical STB layer pattern observed in most controls was only observed in a third (4/12) of the PE placentae. C1q depositions correlated negatively with the soluble regulator FH in the late-onset group (**Table 7**). Calcareous and necrotic areas were intensely stained for FH (**Figure 6K**).

TERMINAL PATHWAY

The terminal complex of C activation was assessed by staining for tissue associated C9. It could represent deposited polymeric MAC or tissue-bound SC5b-9 complexes. Positive staining for C9 was found in distinct regions of the placentae (**Figures 4E–G**). A strong staining was seen in the basal membrane of the STB layer (**Figure 4G**). C9 was also seen in the villous stroma in a non-regular pattern (**Figure 4E**). The most abundant staining for C9 was seen in the fibrinoid and necrotic/calcified areas. There were no differences between the PE and control groups in the C9 staining patterns or intensities (**Tables 4–6**).

MEMBRANE REGULATORS

The membrane-bound regulators of complement MCP, DAF, and CD59 were typically observed in a dual pattern, where both the apical and the basal layer of the STB stained positive. Membrane

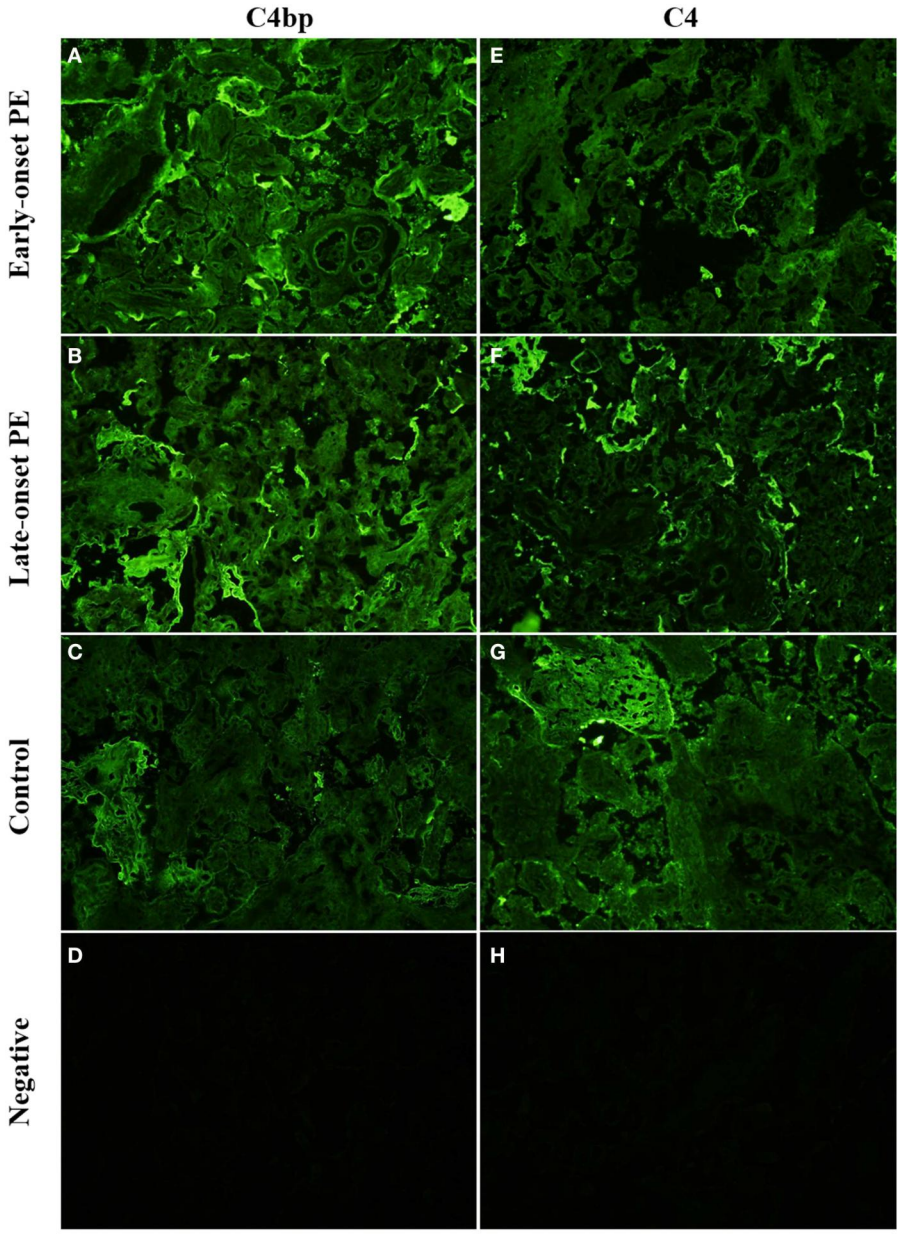

FIGURE 5 | Side-by-side comparison of C4 and C4bp stainings in the same placental samples. C4 is deposited in syncytial knots, visible as bright clusters but also in the syncytia of selected villi. C4bp is observed in syncytial bodies. C4bp is not observed in a circumferential pattern of the syncytium. C4 and C4bp are both observed in fibrinoid structures [here in control panels **(G,C)**]. Top row **(A,E)**: early-onset PE. Middle top row **(B,F)**: late-onset PE. Middle bottom row **(C,G)**: control. Bottom row **(D,H)**: negative control (antibody I omitted). 20× magnification.

regulators were mainly observed in the STB layer and to a lesser extent in the villar endothelium. DAF showed the weakest stainings of the membrane regulators. Unlike the soluble regulators, membrane-bound regulators were not observed in the syncytial bodies (**Figure 1**) nor in apoptotic, necrotic, or fibrinoid structures. No differences in the expression patterns of membrane regulators were observed between PE patients and healthy controls or between the early-onset and late-onset PE (data not shown). The C3b/C4b receptor CR1 (CD35) was not detected in the placentae of the control group or patients (data not shown). This indicates the lack of leukocytes in the placenta.

s-ENDOGLIN

We found s-endoglin to be significantly more abundant in PE pregnancies than in healthy controls (**Table 6**), especially in the late-onset disease group (**Tables 4** and **5**).

In PE, villi in 83% (10/12) of the placentae had distinct and circumferential endoglin deposition on the apical sides of the STB, sometimes penetrating through to the basal layer. Villi with negative staining in the STB were scarce (**Figures 4I,J**). In control placentas, s-endoglin was found mostly in the apical side of the STB layer. 20% of controls had a thin but circumferential deposition pattern on the STB of the villi. While some villi displayed a

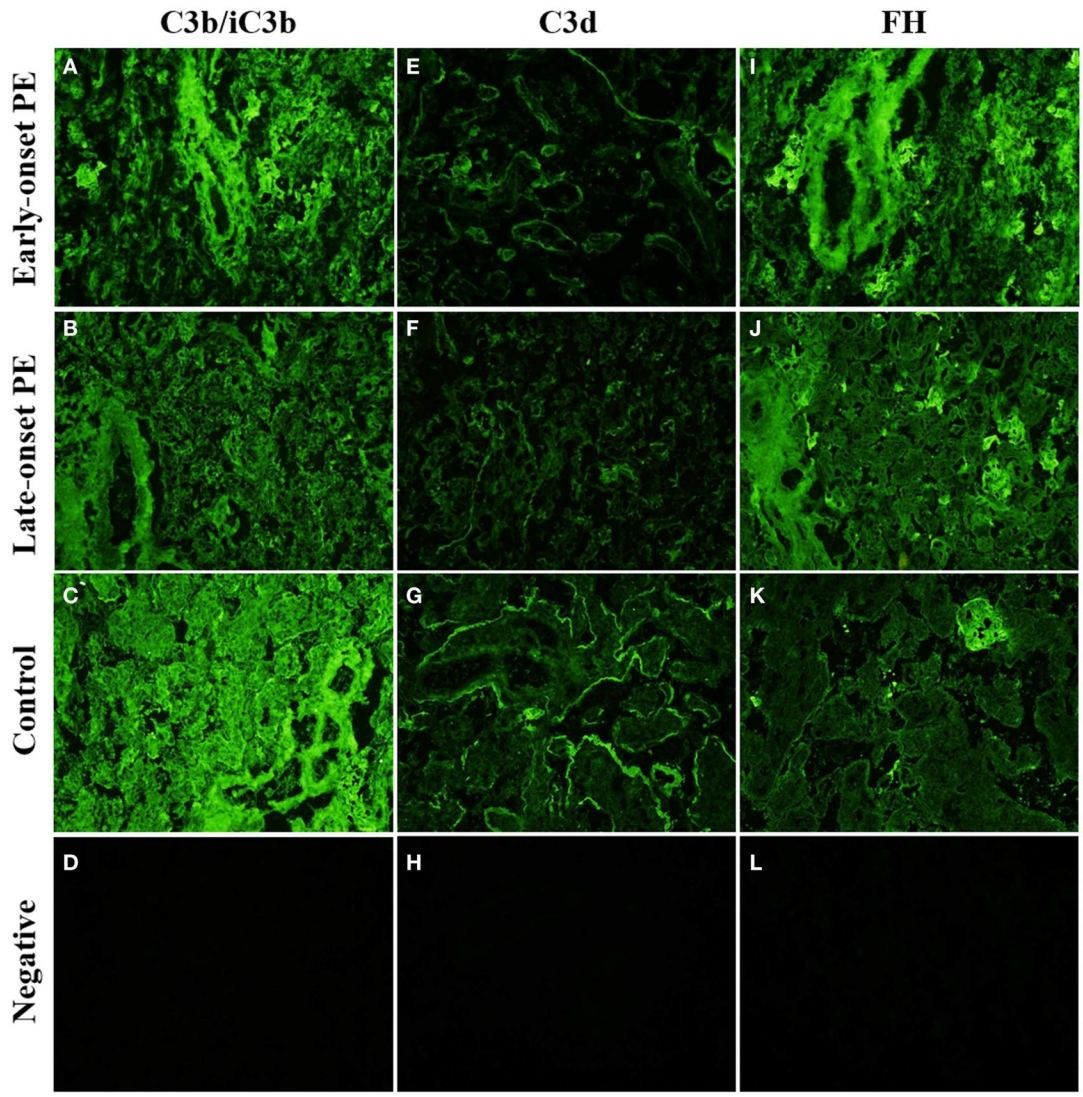

FIGURE 6 | Side-by-side comparison of C3b/iC3b, C3d, and FH staining patterns in the same placentas. C3b/iC3b and FH are observed in stromas of large stem villi [early-onset preeclampsia (PE): **(A,I)** and control: **(C)**] and in fibrinoid structures **(J)**. C3d is typically observed in circumferential pattern of basal membrane of the syncytium **(E–G)**. The circumferential pattern is often interrupted in PE **(E,F)** where physical damage to the syncytium causes increased rate of syncytial shedding. Top row **(A,E,I)**: early-onset PE. Middle top row **(B,F,J)**: late-onset PE. Middle bottom row **(C,G,K)**: control. Bottom row **(D,H,L)**: negative control (antibody I omitted). 20× magnification.

somewhat even positive layer, many villi stained negative or had only a few faintly positive deposits (**Figure 4K**).

DISCUSSION

Differences between women with and without PE were seen in the classical pathway of C activation and in the binding of protective C regulators to the placental structures. The critical differences were mostly observed at the STB layer and in lesions representing injured tissue structures. C1q deposition on the STB was least abundant in the late-onset PE group. Interestingly, in our small study sample we observed that *C4* deficiencies were more common in women with PE compared to women without PE. C4bp was localized to the syncytial bodies, which were more commonly seen in PE. FH was seen in the villi around the STB, although the extent of its binding varied greatly between individuals. In PE patients, less FH in the STB was observed.

Abundant evidence suggests that immunological mechanisms are involved in the various steps of PE pathogenesis. These include an incomplete spiral artery remodeling that causes poor placental development and creates turbulent and constrained blood flow to the villi (17). This in turn will aggravate the physical strain on the placental tissue and may lead to vascular symptoms typical for PE. An abnormal C function could limit the ability of C to maintain its waste disposal function. Accumulation of waste products in the placenta and insufficient repair functions could be related to the pathophysiology of PE.

The overall deposition of C1q was stronger in the early-onset than in the late-onset PE, which may reflect the difference in

Table 5 | Complement and s-endoglin deposition in early- vs. late-onset preeclampsia (PE).

	Type of PE onset	C3b/iC3b			C1q			C4bp			C4		
		Late			Late			Late			Late		
		t	*p*	df	*t*	*p*	df	*t*	*p*	df	*t*	*p*	df
Sum	Early	1.219	0.251	10	**2.273**	**0.046**	**10**	0.176	0.864	9	0.639	0.537	10
Mean	Early	1.297	0.224	10	**3.614**	**0.005**	**10**	0.127	0.902	9	0.364	0.723	10
	Type of PE onset	**FH**			**C3d**			**C9**			**s-Endoglin**		
		Late			Late			Late			Late		
		t	*p*	df	*t*	*p*	df	*t*	*p*	df	*t*	*p*	df
Sum	Early	0.773	0.457	10	0.074	0.943	10	0.857	0.412	10	0.588	0.569	10
Mean	Early	0.722	0.487	10	0.085	0.934	10	0.810	0.437	10	0.511	0.621	10

Mean fluorescence intensity and sum of intensity are compared between early- and late-onset PE and controls. Both sum and mean of C1q differs significantly between the two patient groups.
Bold significant to the 0.05 level.
df, degrees of freedom.

Table 6 | Complement and s-endoglin deposition in preeclampsia (PE) vs. control.

	dg	C3b/iC3b			C1q			C4bp			C4		
		Control			Control			Control			Control		
		t	*p*	df	*t*	*p*	df	*t*	*p*	df	*t*	*p*	df
Sum	PE	−1.417	−0.172	20	−1.695	0.106	20	−0.775	0.448	19	0.848	0.407	19
Mean	PE	−1.366	0.187	20	−1.493	0.151	20	−0.602	0.554	19	−0.664	0.515	19
	dg	**FH**			**C3d**			**C9**			**s-Endoglin**		
		Control			Control			Control			Control		
		t	*p*	df	*t*	*p*	df	*t*	*p*	df	*t*	*p*	df
Sum	PE	0.458	0.652	20	0.045	0.964	20	−0.411	0.685	20	*2.008*	*0.059*	*19*
Mean	PE	0.407	0.689	20	−0.398[a]	0.695	18.961	−0.330	0.745	20	**2.463**	**0.023**	**19**

Pooled PE and controls compared using mean fluorescence intensity and sum of intensity as measurements. Only soluble endoglin is significantly (mean) or borderline (sum) significantly different across the groups.
[a] *Equal variances not assumed.*
Bold significant to the 0.05 level.
Italics borderline significant.
df, degrees of freedom.
dg, diagnosis.

the etiopathogenesis between these two patient groups. Placental dysfunction is typically observed in the early-onset disease. We observed a higher frequency of necrotic large villi and an increased number of fibrinoid necrotic areas in the early-onset PE placentas (2, 18, 19). Importantly, in the early-onset PE placentae, a high-intensity area of C1q was found to lack C9, which indicates that C1q deposition probably does not result in the activation of the terminal pathway in these patients. If C4 deposition is considered indicative of classical pathway activation, missing C1q in the PE patients' syncytium, where C4 deposits were typically observed, suggests that C1q deposition has other functions apart from classical pathway activation in the placenta. The classical pathway of the C system is triggered e.g., by the binding of C1q to immune complexes, CRP and other pentraxins or by intracellular components

Table 7 | Correlations of high-intensity area percentage values between different components per patient group.

	Component 1	Component 2	Pearson's *r*	*p* (2-Tailed)
Early-onset PE	**C1q**	**C9**	**−0.878**	**0.009**
	DAF	C4	0.824	0.023
	C3b/iC3b	s-Eng	0.829	0.021
Late-onset PE	C1q	FH	−0.932	0.021
	C4	C9	0.921	0.026
	C4bp	**C4**	**0.962**	**0.009**
Control	DAF	MCP	0.634	0.049
	C4bp	MCP	−0.659	0.038
	FH	C9	0.671	0.034

Significant correlations to the 0.01 level is indicated in bold, others are significant to 0.05 level.
PE, preeclampsia.

released upon cell damage (20). The possibly elevated plasma CRP levels of PE patients were not reflected in the placental deposition of CRP according to our findings (21).

Our results support the theory, that C1q has an important role in the maintenance of immune tolerance by clearing apoptotic and self-antigens. C1q has an important ability to recognize altered or exposed structures of self thereby leading to their efficient clearance by phagocytes without lysis and inflammation (22). Direct binding of C1q may occur to various structures, such as to phospholipids or vimentin exposed by vascular endothelia during tissue damage (23). Deficiency in C1q is associated with a major insufficiency in the clearance of apoptotic cells. This causes an SLE-like disease often involving glomerulonephritis (24). In PE, a partially similar function for C1q could be envisioned. Continuing stress and, by definition, the temporary existence of placenta may predispose the STB to cellular damage.

High-intensity areas of C1q deposition were negatively correlated with the soluble regulator FH in the late-onset PE group. This could be a reflection of a C1q/FH balance. Our results suggest that C1q could also bind to structures exposed by a turbulent blood flow during PE. These structures could be within the connective tissue, in vascular endothelia or on the trophoblastic cells. It was recently shown that FH can bind independently from and even compete with C1q for binding to apoptotic surfaces and other targets (25). When FH binds directly to the apoptotic surface and tissue debris, it may serve to further dampen the inflammatory response initiated via the classical pathway.

We found an increasing frequency of *C4A* deficiencies with the severity of the PE diagnosis. *C4A* deficiencies are observed in approximately 16% of the Finnish general population (14, 26). We found a 2.5-fold increase in the occurrence of *C4A* deficiency (40%) in the late-onset PE group and a slightly higher frequency in the early-onset group (43%). Because of the small sample size, the studies need to be repeated in a larger material. Based on our interesting preliminary results such a study is underway. While the two C4 proteins, C4A and C4B, have mainly overlapping functions, some differences may also be observed (27). *C4A* deficiencies are often associated with susceptibility to autoimmune diseases (28). Our patients and controls were selected so that patients with immunological diseases that might be associated with *C4A* deficiency, such as SLE, were excluded. Low plasma C4 levels in preeclamptic women have been previously found to be the only abnormality in a panel of C components measured in PE pregnancies and healthy controls (29). The presence of two C4 products with differing functions could explain, why in that study, C4 levels observed in the placenta did not associate with the known deficiency status, as the used antibody does not discriminate between the two forms of C4.

C4bp is a major soluble protein that binds to C4b and regulates the classical pathway C3 convertase. We observed C4bp binding typically and intensely to apoptotic fragments and structures including shed and knotted syncytium. Because of this, it was not possible to quantitatively differentiate if fluorescence intensity levels in relation to disease status were due to the different levels of physical damage of the placenta or to truly different levels of C4bp (30). C4bp is known to bind directly to surfaces of apoptotic and necrotic cells (31, 32). Furthermore, syncytial bodies such as syncytial knots and sprouts might be more common in PE (33). Interestingly, analyses of the high-intensity fluorescence areas showed a strong correlation between C4bp, C4, and C9. This could be indicative of classical pathway activation. Previous reports of C4bp in PE are few and inconclusive. Mellembakken et al. found no difference in C4bp plasma levels of preeclamptics and controls, while Schjetlein et al. reported a decrease in C4bp plasma levels with increasing severity of PE (29, 34). Recently, mutations in C4bp were found to be related to recurrent pregnancy loss in certain patients (10).

Factor H is the most important regulator of the alternative pathway. Generally, FH was observed in abundance in most of the placentae. The absence of STB staining for FH in the PE cases, a trend which was also apparent with other C components, is likely due to the loss of surface negative charge and/or disturbed structure of the STB in the preeclamptic placentae. In clusters of C3b deposition, e.g., in areas of STB damage, FH would also bind. Considering that FH is a soluble molecule, it seems atypical that in 83% of cases and 40% controls, intense FH deposition was observed in the stromas of the tissue. This suggests an increased requirement of the tissue for protection from C attack in majority of the placentae from preeclamptic pregnancies. Interestingly, FH is known to be produced extrahepatically in certain tissues, including the placenta (35). It is likely, that in the placentae with staining of the stroma, the observed FH profile is a heterogeneous combination of the maternal FH localized on the syncytium and fetal FH localized in the villar arterial endothelium

and tissue stromas. At these sites, FH would regulate the alternative pathway.

C3 is the most central component of the C system. We found C3 abundantly in the placenta. In the high-intensity area analysis, C3b/iC3b deposition correlated with C1q and negatively with FH deposition in the early-onset PE patients. This suggests that in the most difficult cases of PE where, the alternative pathway has become activated and, as a result, C3 deposits were observed, regulation by FH had failed to protect the placenta. This relationship was missing in the late-onset PE and control groups. There were no clear differences in the intensity or distribution of C3d between placentas from PE patients and healthy controls. The kinetics of C3d deposition differs from that of C3b and iC3b. C3d is usually found as a remnant from prolonged complement activation, especially in basement membranes.

C3d was localized most clearly to the sub-syncytium basement membrane. There was no difference in the intensity of C3d expression between the control and PE groups. This is in contrast to Sinha et al., who observed that more C3d deposition occurred in the trophoblast basement membrane in PE than in normal placentae, and that the positivity was more marked in the severe PE group than in patients with mild PE (36). In C3-knockout mice, C3 has been found to be needed for successful pregnancy. C3-knockout mice had a lower rate of conception and the fetal reabsorption rate was higher, while the fetal and placental weights were lower in these mice (8). Furthermore, in mice the C3-regulator Crry was found to be crucial to a successful pregnancy (9).

While MCP is an important inhibitor of C activation in the healthy maternal–fetal interface, C4bp apparently has a different role in binding mainly to apoptotic structures and damaged STB. This was reflected by a negative correlation between C4bp and MCP intensity in the controls. The correlation between MCP and DAF (CD55), which was observed in the high-intensity areas of the control placentae showed that these regulators synergize each other in the regulation of C activation in the third trimester placenta. They are probably both needed for the control of C activation on the syncytium. Membrane-bound regulators of complement DAF, MCP, and CD59 have been observed in the healthy first trimester and term placentae, where they serve to protect the developing placenta from C attack (5, 37, 38). In general, no differences in the CD59 patterns or abundance were observed between the preeclamptics and controls.

Increased endoglin staining was observed in PE samples. Our results corroborate the findings of Sitras et al. and Nishizawa et al. who both found increased levels of endoglin in PE, but could not differentiate late- and early-onset patient groups with regard to endoglin expression (39, 40). s-eng is a placenta-derived TGF-beta co-receptor known to be elevated in PE although reports of circulating s-eng levels correlating with disease severity are not consistent (16). Since it is known that s-eng is more abundant in PE than in healthy pregnancies, we used it as a positive control to verify the ability of our methodology to detect changes in immunohistochemical stain pattern and intensity between PE and control placental samples.

CONCLUSION

Here, we have described for the first time the expression of a large panel of C system components in the placentae of PE pregnancies. It is apparent that complement is involved in multiple ways in both normal pregnancy as well as in PE.

A notable difference between two soluble C regulators was observed. C4bp was found to bind directly to apoptotic syncytial structures while fetal FH apparently provides an overall, broad scale protection to the placental tissue.

Correlation of MAC deposition with the classical pathway activating components in the patient groups and negative correlation in the controls may be indicative of C regulation breakdown and/or uncontrolled classical pathway activation in PE.

In our small cohort partial *C4A* deficiency was more frequent in patients than controls. The high incidence of *C4A* deficiency in PE might bear functional importance and the issue needs to be examined in a larger material.

AUTHOR CONTRIBUTIONS

Anna Inkeri Lokki and Jenni Heikkinen-Eloranta did the laboratory work for the project, analyzed the data, and drafted the manuscript. Jenni Heikkinen-Eloranta described the patient material with help from Hannele Laivuori and Terhi Saisto. Hannele Laivuori is the head of the FINNPEC board and together with Terhi Saisto and Jenni Heikkinen-Eloranta she collected the samples and clinical data. Hanna Jarva and Seppo Meri are experts in the complement system and participated in planning the laboratory methodology (Hanna Jarva) and providing the materials and laboratory space (Seppo Meri). Marja-Liisa Lokki is a specialist in the field of MHC genetics and planned, analyzed, and helped to interpret the C4 genetic portion. Hannele Laivuori and Seppo Meri came up with the study question planned the project in collaboration with Anna Inkeri Lokki and Jenni Heikkinen-Eloranta.

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PLOS ONE

RESEARCH ARTICLE

Genetic Analysis of Membrane Cofactor Protein (CD46) of the Complement System in Women with and without Preeclamptic Pregnancies

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Abstract

Preeclampsia is a common disorder of pregnancy characterized by endothelial dysfunction. It may be life-threatening for the mother and fetus in severe cases. Dysregulation of the complement system has been suggested to predispose women to preeclampsia. Complement is part of the innate and adaptive immune systems and potentially capable of causing inflammation and tissue damage. Membrane cofactor protein MCP (CD46) is among the potent complement regulators that have recently been linked to a severe form of preeclampsia with or without an underlying autoimmune phenotype. Mutations in *CD46* predispose to thrombotic microangiopathy with endothelial cell dysfunction. The exome of *CD46* were sequenced in 95 Finnish women with severe preeclampsia. Genetic variations discovered in the full exome were compared to those observed in 95 control women who did not develop preeclampsia. Because A304V (rs35366573) was associated with preeclampsia in one previous study, we sequenced the transmembrane region including the A304V variant and part of the cytoplasmic tail in 95 additional controls. We did not discover any association between A304V or other *CD46* SNPs and preeclampsia. This study describes a carefully characterized cohort of severely preeclamptic Finnish women and found no potentially predisposing variants in *CD46*. However, it is possible that other genetic components of the complement system may affect the pathogenesis of severe preeclampsia and related diseases.

Introduction

Preeclampsia is a common (3–5%) disorder of pregnancy. It increases perinatal mortality five-fold [1]. The onset and clinical course of preeclampsia is unpredictable. It often necessitates preterm delivery, which carries the risk for complications of prematurity.

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Preeclampsia is also associated with an increased risk of cardiovascular disease in later life of both the mother and the fetus [2,3]. Although the disease originates early in pregnancy, the defining symptoms, newly-onset hypertension and proteinuria, develop in the latter half of pregnancy. Currently, no reliable predictive test for preeclampsia is known and no cure other than delivery is available.

The complement system is an integral part of the innate immune system consisting of phylogenetically ancient processes of pathogen recognition and self-/non-self-discrimination. In a normal pregnancy the complement system becomes activated at a low level [4]. Activation of the terminal complement pathway leads to formation of the membrane attack complex (MAC) and possible destruction of the target cell. Complement can be activated via three independent pathways. The lectin pathway (LP) is activated by mannose-binding lectin (MBL) and by ficolins. It has previously been studied in association with preeclampsia with contradictory results [4,5]. The classical pathway (CP) is activated by the C-reactive protein (CRP) or by immune complexes, such as antibodies bound to microbes or autoantibodies bound to self-antigens. The role of a microbial trigger for inflammation during pregnancy as a causative agent in preeclampsia has been the subject of a lengthy debate, where no definitive conclusions have yet been achieved [6–9]. An important role for the alternative pathway (AP) of complement in abnormal pregnancy was suggested by animal studies over a decade ago, when Crry, the functional murine homologue of human complement inhibitors membrane cofactor protein (MCP) and decay accelerating factor (DAF), was knocked out causing infertility due to alternative pathway activation [10]. Recently, it was shown that an elevated level of alternative pathway activation occurs also in human pregnancies, where severe preeclampsia develops [11]. Activation of the complement system must be rigorously regulated in order for normal placentation to occur and for the placenta to be protected from tissue destruction, thrombosis and antiangiogenic factors [12,13]. MCP is a widely expressed complement regulator that inhibits AP, LP and CP. It binds to both C3b (AP) and C4b (CP) and acts as a cofactor for their inactivation by the C3b/C4b inactivator enzyme factor I [14,15]. MCP is a type 1 membrane bound protein consisting of an intracellular tail region with several variant structures [7] a single transmembrane α-helical region, a short region of unknown function (U), a collar STP- rich region with several variant structures, and finally four extracellular Complement Control Protein (CCP) domains carrying the cofactor activity (Fig. 1) [15,16].

MCP is encoded by *CD46* located in the Regulators of Complement Activation gene cluster on chromosome 1q32.2. Apart from its role as a complement inhibitor on cell membranes MCP can act as a receptor for certain pathogens such as the measles virus (Edmonston strain) [17], human herpesvirus 6 [18] and *Neisseria gonorrhoeae* [19]. MCP has also been suggested to have role in the fertilization of the human egg [20]. Notably, several mutations both in the CCPs as well as in the transmembrane region are linked to atypical hemolytic uremic syndrome (aHUS) [21,22].While we initiated our studies on the AP regulatory genes in preeclampsia, MCP among other complement regulators received considerable attention as potential target for mutations in preeclampsia [12].

We hypothesized that variation in complement regulation might be one underlying reason for severe preeclampsia in patients who developed heavy proteinuria. The objective of this study was to investigate whether sequence variations in the *CD46* promoter, splice sites and exome are present in Finnish women with and without preeclampsia. Because A304V (rs35366573) was associated with preeclampsia in one previous study [12], we studied this variant in a larger group of controls.

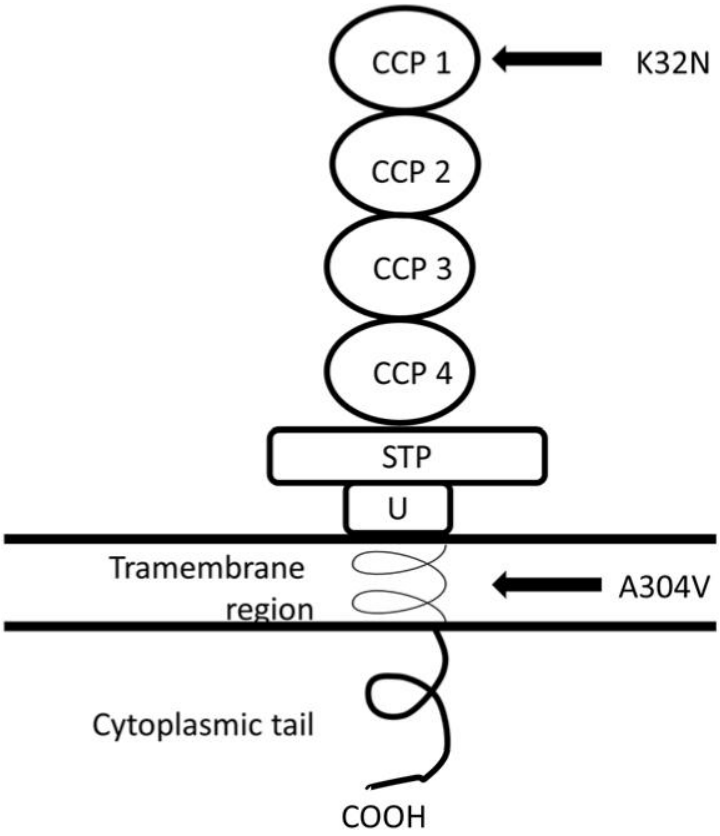

Fig 1. The schematic structure of the membrane cofactor protein (CD46). The positions of the amino acid changing single nucleotide polymorphisms described in Salmon *et al*. [12] and in this study are depicted. Inspired by Fang *et al*. [22].

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Methods

STUDY POPULATION

The participants are a subgroup of the FINNPEC (The Finnish Genetics of Preeclampsia Consortium) study cohort. Briefly, at the time of publication, the cohort comprises 1090 women diagnosed with preeclampsia in their singleton pregnancy and 930 women without preeclampsia symptoms in their singleton pregnancy (the control group). Preeclampsia was defined as hypertension and proteinuria occurring after 20 week of gestation. Hypertension was defined as a systolic blood pressure $\geq$ 140 mmHg, and/or a diastolic blood pressure $\geq$ 90 mmHg. Proteinuria was defined as the urinary excretion of $\geq$0.3 g protein in a 24-hour specimen, or 0.3g/L, or $\geq$1+ reading on dipstick in a random urine determination at least twice with no evidence of a urinary tract infection. Intrauterine growth restriction (IUGR) was defined as birth weight below-2 SD according to the Finnish standards [23].

For this study we selected a subset of 95 primiparous women with severe preeclampsia (a systolic blood pressure $\geq$160 mmHg or a diastolic blood pressure $\geq$110 mmHg or proteinuria $\geq$5 g protein in a 24-hour specimen). Because the prominent mutations of *CD46* are known to associate with disorders involving kidney function, we included preeclamptic women with heavy proteinuria in this study. They had no autoimmune diseases. Twenty-six patients (27%)

had early-onset preeclampsia (diagnosis before 34 weeks of gestation). A control group comprised 190 women randomly selected from the original control group. None of the controls had autoimmune diseases. The clinical characteristics of the two groups are shown in Table 1.

ETHICS STATEMENT

All subjects provided a written informed consent and the FINNPEC study protocol was approved by the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (permit number 149/E0/07).

LABORATORY METHODS

DNA was extracted from 10 ml EDTA whole blood stored at-20C° (after initial freezing period at-80C° to prevent formation of icicles and molecular degradation) using Chemagic Magnetic Separation Module I (Chemagen, PerkinElmer, Baesweiler, Germany) automatic DNA extraction protocol as provided by the manufacturer. Extracted DNA was used at final concentrations of 20 ng /μl and 30 ng /μl.

Exomic sequencing inclusive of flanking intronic regions was carried out using 15 pairs of primers as listed in S1 Table. Promoter region 240 bp upstream of exon 1 was also sequenced. Primers were designed and tested by using Primer3 and GenomeTester softwares [24,25]. PCR was carried out using Mytaq (Bioline, London, UK) polymerase enzyme and buffer following the manufacturer's protocol. PCR products were purified using ExoSAP-IT (GE Healthcare Life Science, UK) shrimp phosphatase alkaline product and the purified DNA fragments were sequenced by Big Dye Terminator v3.1 Cycle enzyme and buffer (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's protocol. The sequencing reaction product was purified by Performa DTR v3 filterplates (Edge BioSystems, Gaithersburg, MD, USA) and analyzed using ABI3730xl capillary electrophoresis sequencer (Applied Biosystems, Carlsbad, CA, USA).

The raw data was analyzed using Sequencher 8 software (Applied Biosystems, Carlsbad, CA, USA) and the detected polymorphisms were verified using SNPper [26] and NCBI databases.

STATISTICAL METHODS

CD46 sequencing results were analyzed for disease association in PLINK [27]. Association by individual SNPs and association in terms of different genotypic models were evaluated by Fisher's Exact test. The functionality of amino acid changing SNPs was evaluated using PolyPhen-2 [28] and SIFT [29]. In SIFT, scores < 0.05 or sometimes < 0.1 are considered indication of a deleterious mutation [30]. An independent values t-test was used to compare means of clinical characteristics between patients and controls in IBM SPSS statistics version 22 (IBM corp.).

Results

A total of 15 SNPs (Table 2), including three novel ones (Table 3), were observed in *CD46*. The alleles and minor allele frequencies (MAF) in patients and controls are presented in Table 2. Heterozygosity for the A304V variant (rs35366573) was observed in 12% (11/95) of cases and 11% (21/188) of controls (OR = 0.943, 95% CI = 0.450, 1.978). In addition, one control was homozygous for A304V. A304V was predicted to be benign (Polyphen2 score 0.011/1) and tolerated (Sift: 0.19). The K32N variant (rs150429980), an amino acid change in the functional part of the MCP molecule, in the most membrane distal CCP, was observed in a heterozygous form in one patient and in one control (OR = 1, 95% CI = 0.062, 16.11, S2 Table). K32N was ambivalently predicted to be probably damaging (Polyphen2 score 0.996/1, sensitivity 0.55, specificity 0.98) or possibly tolerated

Table 1. Clinical characteristics of the study population.

	Severe preeclampsia n = 95 Mean (SD) N (%)	Controls n = 190 Mean (SD) N (%)	Difference between patients and controls
Age (years)	29.0 (4.55)	30.8 (4.87)	$p = 0.003^{\dagger}$
Body mass index (kg)	24.2 (4.92)	23.9 (3.72)	NS†
Primipara	95 (100%)	104 (55%)	$p < 0.001^{\ddagger}$
Early-onset preeclampsia (diagnoses <34 weeks of gestation)	26 (27%)	0	$p < 0.001^{\ddagger}$
Systolic blood pressure (mmHg)	173 (15.36)	125 (12.94)	$p < 0.001^{\dagger}$
Diastolic blood pressure (mmHg)	112 (6.92)	83 (7.99)	$p < 0.001^{\dagger}$
Proteinuria (g, 24-hour specimen)	5.8 (4.49)	*	
Gestational diabetes	2 (2%)	10 (5%)	$p = 0.005^{\ddagger}$
Pre-gestational diabetes (type 1 diabetes)	0	3 (2%)	NS‡
Pre-gestational hypertension	1 (1%)	3 (2%)	NS‡
Gestational hypertension	0	7 (4%)	$p = 0.043^{\ddagger}$
Weeks of gestation at delivery	36.2 (2.89)	39.9 (1.58)	$p < 0.001^{\S\dagger}$
Preterm birth (delivery before 37 weeks of gestation)	39 (42%)	6 (3.2%)	$p < 0.001^{\ddagger}$
Birthweight (g)	2582 (733.86)	3622 (474.27)	$p < 0.001^{\S\dagger}$
Intrauterine growth restriction (<-2 SD)	18 (19%)	1 (<1%)	$p < 0.001^{\ddagger}$

The 95 subgrou of controls did not differ statistically from the 190 control pool. The significance level is 0.05.

NS—not significant

§ Equal variances not assumed

* One individual (0.5%) in the control group had gestational proteinuria.

† T-test

‡ Mann-Whitney U test

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Table 2. The observed single nucleotide polymorphisms (SNPs) and minor allele frequencies (MAF) in the genotyped cases and controls.

CHR	SNP	MAF cases	MAF controls	N cases	N controls	Location
1	rs2796268	0.453	0.457	95	94	promoter
1	rs41266397	0.069	0.074	95	95	promoter
1	rs150429980	0.005	0.005	95	94	exon 2 K32N
1	rs12126088	0.011	0.000	95	94	exon 4 synonymous
1	rs41258244	0.069	0.074	95	94	exon 5 synonymous
1	rs2724374	0.247	0.200	95	95	intronic
1	rs35366573	0.058	0.061	95	188	exon 11 A304V
1	NewSNP1	0.000	0.005	92	97	intronic
1	NewSNP2	0.005	0.000	95	94	cytoplasmic tail
1	NewSNP3	0.005	0.000	95	187	cytoplasmic tail
1	rs7144	0.452	0.457	95	94	cytoplasmic tail
1	rs193023975	0.005	0.000	95	94	cytoplasmic tail
1	rs185457983	0.000	0.005	95	94	cytoplasmic tail
1	rs14374	0.053	0.021	95	95	cytoplasmic tail
1	rs1237	0.069	0.042	95	95	cytoplasmic tail

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Table 3. Details of the new single nucleotide polymorphisms (SNPs).

SNP ID	Allele 1	Allele 2	Genomic position*
NewSNP1	G	A	207963561
NewSNP2	G	C	207967178
NewSNP3	A	G	207967697

* according to build GRCh37.p10

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(Sift: 0.13). One new SNP was located near exon 13, being observed in one control. Two of the new SNPs were located in exon 14. Both were observed once in a different patient (Fig. 2, Table 3).

Discussion

In this study we did not find any sequence variants in promoters, exomes and flanking regions of *CD46* associated with preeclampsia. Preeclampsia is known to be a clinically variable and etiologically heterogeneous disease [31]. Preeclamptic patients with heavy proteinuria were selected for the project, because several previous studies pointed towards functional problems of the kidney in association with complement activation and particularly with *CD46* mutations, as observed in aHUS [17,21,22,32]. The similar nephrological symptoms between aHUS and preeclampsia led us to pursue a common cause to these two conditions.

Interestingly, Salmon and co-workers reported *CD46* mutations as genetic defects associated with preeclampsia [12]. In the PROMISSE cohort consisting of 250 pregnant patients with *systemic lupus erythematosus (SLE)* and/or antiphospholipid antibody (*APL Ab*) syndrome they found an increase from 2.5% to 7% in A304V heterozygosity in women, who developed preeclampsia. They replicated their finding in 59 women with preeclampsia and/or the *HELLP* (hemolysis, elevated liver enzymes, low platelet count) syndrome and 143 controls without autoimmune diasease [12]. In the 59 patients, Salmon *et al.* observed a higher MAF (minor allele frequency 3.4%) than what was seen in ethnically and geographically matched controls (1.4%, including 1 homozygous individual) [12]. Our results with a larger study population do not support the previously reported findings. In the present study, A304V variant was equally prevalent in the two groups. Furthermore, we discovered an individual homozygous for A304V among the healthy controls. MAF for A304V was 5.4% in 3316 Finnish individuals of the SISu (Sequencing initiative Suomi) data resource (www.sisuproject.fi). While NCBI reports heterozygosity in different populations to range between 2–4%, with MAF of 0.6–2.2%, the highest values being recorded in European populations, our result together with the SISu data suggests, that an even higher MAF is typical of the general Finnish population. In light of this evidence it seems unlikely that the increase in MAF from 2.1% (general population) or 1.4% (healthy geo-ethnically matched pregnant controls) to 3.7% (PROMISSE with preeclampsia) or 3.4% (Utah Severe preeclampsia/HELLP cohort) alone would bear functional consequences for the development or progression of preeclampsia, as previously suggested [12]. Furthermore, preeclampsia being a heterogenous disease, different subtypes such as HELLP may not be compatible in etiological comparison. Our data set had no patients with severe immunological diagnoses such as SLE. The patients with severe preeclampsia from the study by Salmon *et al.* are comparable to our data set [12].

A304V variant is located in the transmembrane region of the molecule (Fig. 1). The possible functional role is thus not directly related to interaction with C3b and C4b, which are known to bind to extracellular CCP regions. The mechanism of decreased alternative pathway regulation associated with A304V is thus not clear [22,33]. We did not find indication of disease

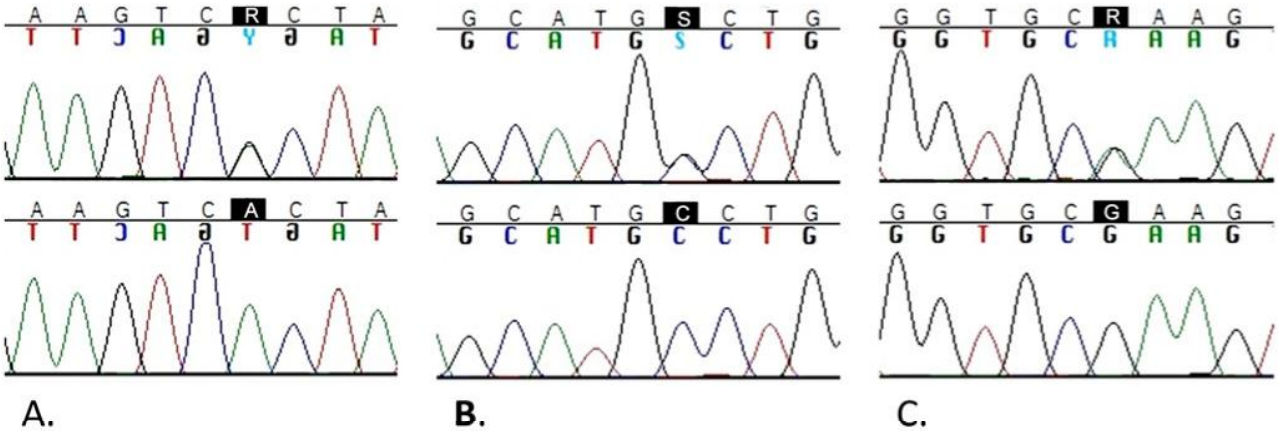

Fig 2. The sequences of the newly discovered SNPs near exon 13 and in exon 14 of the *CD46*. Panel A: New SNP1 G/A heterozygote (top) and A/A homozygote (bottom). Panel B: New SNP2 G/C heterozygote (top) and C/C homozygote (bottom). Panel C: New SNP3 A/G heterozygote (top) and G/G homozygote (bottom).

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relatedness of A304V in our bioinformatics analyses in a majority of the transcripts, i.e. splice variants included in the software analyses.

Salmon et al. found K32N in a patient affected by SLE with a history of repeated pregnancy loss, fetal death and preeclampsia. This amino acid substitution from basic lysine to neutral asparagine causes a 4x decrease in the C4b cleavage promoting activity of MCP [12]. In our material K32N was observed in a heterozygous form in one preeclamptic patient and one control. SISu reports the MAF of K32N to be 0.03% in 3325 Finnish individuals. In the bioinformatics analyses the possible functional effects of K32N were controversial. While SIFT predicted K32N to be tolerated, Polyphen2 predicted it to have deleterious changes for the protein function in all available transcripts.

Preeclampsia being a clinically variable and heterogeneous disease, it is always a challenge to pinpoint an association to link single gene variant to a particular subphenotype. The missing association between *CD46* mutations and preeclampsia may also be attributed to incomplete penetrance of complement mutations in preeclampsia. Furthermore, the multifactorial background is another likely explanation for missing association in a complex disease like preeclampsia. The present study does not account for the effect of disease specific isoforms in preeclampsia. While several splicing isoforms of *CD46* have been known to exist for a long time recent work suggests a disease specific role for the occurrence of *CD46* isoforms [7]. Exons 13 and 14 code for the large cytoplasmic tail section of the molecule, which is variably included or spliced out from the functional isoforms of the protein (Fig. 1) [21]. The functional effects of the new SNPs discovered in these exons fundamentally depend on whether they are included in the isoform in question.

As discussed above, the complement system has an essential role in pregnancy. The overriding hypothesis is that excessive and uncontrolled activation of the maternal complement system compromises the placenta and ultimately the fetus [12,13]. Accordingly, it is not likely that malfunctioning maternal MCP, being a cell-bound regulator of the complement system, would have a fundamental effect on the integrity of tissues of fetal origin such as the placenta. Maternal activators and fetal regulators are the more obvious molecules of interest in future studies.

In this study we have explored the genetic polymorphism of *CD46* in preeclampsia. Three novel rare variants were discovered but neither they nor other polymorphisms of interest were associated with severe preeclampsia. Studies on functional *CD46* isoforms in preeclampsia may provide further insight into the possible role of MCP and complement-mediated injury in the pathogenesis of severe preeclampsia.

Supporting Information

S1 Table. Used primers.
(DOCX)

S2 Table. Association as determined by Fisher's exact.
(DOCX)

Acknowledgments

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The Sequencing Initiative Suomi (SISu) project (www.sisuproject.fi) is an international collaboration between research groups aiming to build tools for genomic medicine. These groups are generating whole genome and whole exome sequence data from Finnish samples and provide data resources for the research community. Key groups of the project are from Universities of Eastern Finland, Oulu and Helsinki and The Institute for Health and Welfare, Finland, Lund University, The Wellcome Trust Sanger Institute, University of Oxford, The Broad Institute of Harvard and MIT, University of Michigan, Washington University in St. Louis, and University of California, Los Angeles (UCLA). The project is coordinated in the Institute for Molecular Medicine Finland at the University of Helsinki.

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Author Contributions

Conceived and designed the experiments: AIL. Performed the experiments: AIL. Analyzed the data: AIL TAV. Contributed reagents/materials/analysis tools: HL SM. Wrote the paper: AIL TAV HL SM. Came up with the study question: SM. Together with FINNPEC collected the patient material and provided the clinical database: HL.

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Analysis of Complement *C3* Gene Reveals Susceptibility to Severe Preeclampsia

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Preeclampsia (PE) is a common vascular disease of pregnancy with genetic predisposition. Dysregulation of the complement system has been implicated, but molecular mechanisms are incompletely understood. In this study, we determined the potential linkage of severe PE to the most central complement gene, *C3*. Three cohorts of Finnish patients and controls were recruited for a genetic case-control study. Participants were genotyped using Sequenom genotyping and Sanger sequencing. Initially, we studied 259 Finnish patients with severe PE and 426 controls from the Southern Finland PE and the Finnish population-based PE cohorts. We used a custom-made single nucleotide polymorphism (SNP) genotyping assay consisting of 98 SNPs in 18 genes that encode components of the complement system. Following the primary screening, *C3* was selected as the candidate gene and consequently Sanger sequenced. Fourteen SNPs from *C3* were also genotyped by a Sequenom panel in 960 patients with severe PE and 705 controls, including already sequenced individuals. Three of the 43 SNPs observed within *C3* were associated with severe PE: rs2287845 ($p = 0.038$, OR = 1.158), rs366510 ($p = 0.039$, OR = 1.158), and rs2287848 ($p = 0.041$, OR = 1.155). We also discovered 16 SNP haplotypes with extreme linkage disequilibrium in the middle of the gene with a protective ($p = 0.044$, OR = 0.628) or a predisposing ($p = 0.011$, OR = 2.110) effect to severe PE depending on the allele combination. Genetic variants associated with PE are located in key domains of C3 and could thereby influence the function of C3. This is, as far as we are aware, the first candidate gene in the complement system with an association to a clinically relevant PE subphenotype, severe PE. The result highlights a potential role for the complement system in the pathogenesis of PE and may help in defining prognostic and therapeutic subgroups of preeclamptic women.

Keywords: preeclampsia, complement, *C3*, association study, gene regulation, genetic risk, pregnancy complication, innate immunity

INTRODUCTION

Preeclampsia (PE) is a serious vascular complication of pregnancy, which may lead to a life-threatening multi-organ dysfunction and a convulsive condition, eclampsia (1). PE affects 3–5% of pregnancies in all ethnic groups. The development and progression of the disease are unpredictable with delivery being the only effective cure.

Preeclampsia has been the subject of numerous genetic studies and several associating single nucleotide polymorphisms (SNPs) have been identified. Among the genes where associating SNPs have been described are genes linked to hypertension and vascular and metabolic disease (2–4), all diseases whose risk is increased in the later life of PE patients (5). Furthermore, genes encoding for proteins involved in the immunological processes have also been found to harbor SNPs that predispose patients to PE (6, 7).

Pregnancy is the ultimate immunological paradox, where the maternal immune system must accommodate to protect the mother and growing fetus from pathogens while allowing the semiallograft fetus to persist and thrive. PE is a vascular disease that involves poor placentation (8), especially in the severe and/or early onset (diagnosis or delivery <34 weeks of gestation) forms of the disease, where immunological mechanisms have been implicated (9, 10). Among the immunological effector mechanisms, inadequate control of the maternal complement system has been suggested to contribute to the etiology of PE (11, 12).

The complement system is a part of the immune system that is involved in generating inflammation and mediating the clearance of microbes and injured tissue materials. It can be activated by the classical, the lectin, or the alternative pathway, which proceed stepwise in a controlled cascade of interactions between surface-bound and soluble proteins in the serum. Complement C3 is the central component of all activation pathways. It is among the most ancient components of innate immunity that has evolved over 1,000 million years ago (13). Indeed, the functional domains of the human C3 are conserved in corals and Cnidarians (14, 15). The ancient evolutionary attribute of C3 and its abundance in the human serum indicate its important role as the key component of immunity against infection and in the discrimination between self and non-self (16).

C3 is a large protein formed by pair of disulfide-linked α- and β-chains and 13 individual domains. In shape, it is a "robot"-like molecule that has eight macroglobulin domains "the body," a linker (LNK), the C3a anaphylatoxin, an arm-like region with the "C1r/s, UEGF, BMP1" (CUB), a thioester-containing domain (TED), an N-terminal domain (α′NT), and the "head" (C345C) linked to the body with an anchor (17). The domains are encoded by 41 exons of the *C3* gene. When C3 is activated to C3b, an internal thioester bond is disrupted allowing covalent attachment of C3b to target surfaces. Subsequently, factor B binds to the MG2 and CUB domains of C3b (18). A C3 convertase, C3bBb, is formed when factor D activates C3b-bound factor B to breakdown product. Thereafter, C3bBb cleaves new C3 molecules to C3b to release anaphylatoxic C3a fragments to the circulation (19). The main inhibitors of C3 activation, factor H, decay accelerating factor (CD55), and CR1 bind partially to the factor B-binding site to prevent or disrupt C3bBb formation (17, 20).

Mao et al. showed that alternative pathway complement activation is the key mechanism for reproductive failure in complement inhibitor deficient (*Crry*$^{-/-}$) mice (21). Recently, it was shown that alternative complement pathway becomes activated also in human pregnancies, where severe PE develops (11). Successful treatment of a patient suffering from HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count), a life-threatening complication of PE, by eculizumab, a targeted inhibitor of complement protein C5, demonstrated that the complement system could provide a promising target for drug development in severe PE (22). C5 is the initiator of the final stages of complement activation, i.e., the lytic terminal pathway.

We have looked for SNP association with severe PE among 18 genes coding for the complement system. The most promising associations were found in *C3*, where linkage both to individual SNPs and to a distinct haplotype, was observed. *C3* was thus subsequently chosen for detailed capillary sequencing of its exons and promoter regions (PROMs) in women with severe PE and controls with non-PE pregnancies.

MATERIALS AND METHODS

This case-control study was conducted at the Department of Medical and Clinical Genetics and Institute for Molecular Medicine Finland in the University of Helsinki. The number of women and methods of genotyping in each stage of the study are described in detail in the work flow chart (**Figure 1**). All subjects provided a written informed consent in accordance with the Declaration of Helsinki. Study protocols were approved by the local Ethical Committees, specifically, for the FINNPEC study, ethical approval has been obtained from the Coordinating Ethics Committee, Hospital District of Helsinki, and Uusimaa, for the Finnish population-based PE cohort was approved by the ethics committee of the Finnish Red Cross Blood Service and by the Ministry of Social Affairs and Health and for Southern Finland PE study was approved by the Ethics Committee of the Department of Obstetrics and Gynaecology at Helsinki University Central Hospital.

Selection of Study Subjects and Diagnostic Criteria

Study subjects were selected from three Finnish PE cohorts; The Finnish population-based PE cohort (23), The Southern Finland PE cohort (23), and the Finnish Genetics of PE Consortium (FINNPEC) cohort (24) (Table S1 in Supplementary Material). Only women with a singleton pregnancy were included in the study. Except for three women from the Southern Finland PE cohort, where data on subjective symptoms were not available, all women with PE met the criteria of severe PE (23). Severe PE was defined as having repeatedly maximum systolic blood pressure (BP) ≥ 160 mmHg and/or maximum diastolic BP ≥ 110 mmHg or proteinuria ≥5 g/day or significant subjective symptoms in a woman diagnosed with PE according to the American College of Obstetricians and Gynecologists (25).

The clinical characteristics of all women whose samples were used for sequencing and Sequenom genotyping are described in **Table 1**. Body mass index was defined as the pre-pregnancy

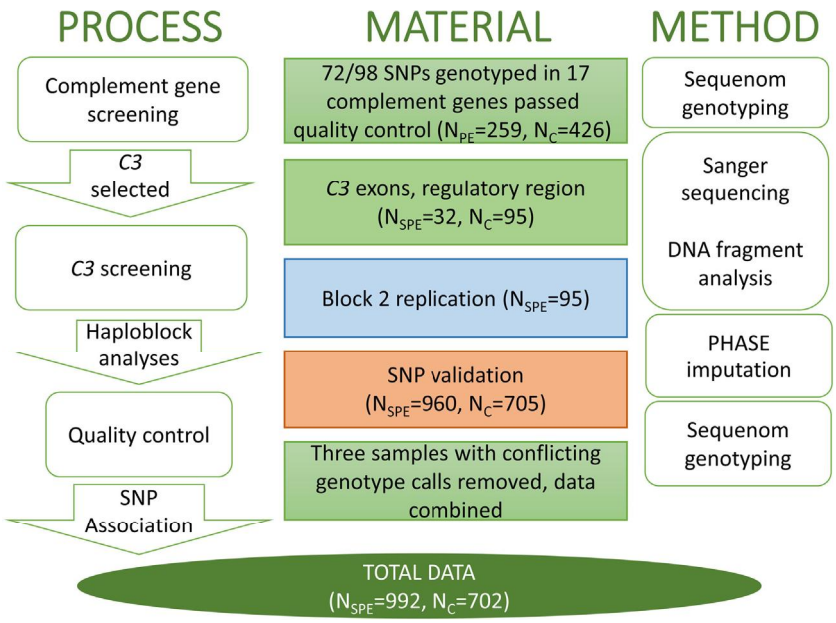

FIGURE 1 | The workflow of C3 project with the analysis process and appropriate method in parallel. The single nucleotide polymorphisms (SNPs) that were assessed in each stage are identified by the respective color in the method panel in **Figure 2**. SPE, severe preeclampsia.

TABLE 1 | Clinical characteristics of the Southern Finland cohort (*N* = 32, data missing for five individuals) and the Finnish Genetics of Preeclampsia (FINNPEC) cohort participants in *C3* sequencing and genotyping.

	Controls (*N* = 702)	Severe preeclampsia (PE) (*N* = 991)	*p*-Value* (compared to controls)	Severe PE	*p*-Value* (compared to controls)
				The Sanger sequencing stage (*N* = 27) median (25th, 75th percentile)	
Age, years	29.5 (26, 33)	31 (27, 35) *N* = 986	<0.001	31 (25, 33)	0.742
Pre-pregnancy body mass index, kg/m^2	23 (20.8, 25.9)	24 (21.3, 28) *N* = 988	<0.001	22.5 (20.7, 24)	0.132
Highest systolic blood pressure (BP), mmHg	125 (118, 133)	171 (161, 184)	<0.001	170 (160, 180)	<0.001
Highest diastolic BP, mmHg	82 (78, 87)	112 (107, 118)	<0.001	105 (100, 110)	<0.001
Proteinuria (DU-prot, diurnal collection sample), g/d		4.2 (1.8, 7.1) *N* = 927	NA	5 (1.7, 15.2)	0.002
Proteinuria (U-prot, single sample), g/l, median (max, min)[a]		1.3 (0.7, 3.2), *N* = 13	0.189	NA	NA
Proteinuria measured by dipstix *N* (% positive: +, ++, +++)[b]	23 (3)	54 (5.4)	0.03	NA	NA
Primipara *N* (%)	377 (54)	733 (73)	<0.001	31 (100)	0.017
Birth weight SD score	0 (–0.6, 0.7) *N* = 700	–1.3 (–2.0, –0.5) *N* = 990	<0.001	–1.7 (–2.3, –0.7)	<0.001
Gestational age at birth, weeks	40 (39, 41)	37 (34, 38)	<0.001	36 (31, 38)	<0.001

*Mann–Whitney U (continuous) or χ^2 (categorical), Fisher's exact for small groups $N < 5$.

[a]Among those with no diurnal protein available.

[b]Among those with no quantitative (diurnal or single sample) protein measurement.

Values for continuous variables are median (25, 75 percentiles) unless otherwise indicated, the number of subjects is indicated where data are not available for all participants. Proteinuria was also observed in 11 controls, who did not meet the diagnostic criteria of PE.

weight in kilograms divided by height in meters squared (kg/m^2). Pregnancy weight and height measures were obtained from the antenatal charts. Relative birth weight SD units (*Z*-score) were calculated according to Finnish standards (26).

Subjects in Complement Genotyping

The SNP genotyping was performed in 259 PE women and 426 non-PE controls. The PE women and controls were selected from the Finnish population-based PE cohort and the Southern Finland PE cohort with preference on severity of the disease.

Subjects in *C3* Sequencing and Microsatellite Analysis

We performed *C3* sequencing and the microsatellite analysis of the upstream regulatory motif in 32 severe PE women from the Southern Finland PE cohort (**Table 1**) and 95 non-PE controls

from the FINNPEC cohort. These data were also used in the relative extended haplotype homozygosity (REHH) statistics.

Subjects in *C3* Replication by Sequencing

Ninety-five women with severe PE from the FINNPEC cohort were selected for the second stage of sequencing, which involved re-sequencing the middle part of the gene indicated by blue in **Figure 2**. The replication sequencing data were used in the REHH statistics.

Subjects in Replication by Sequenom Genotyping

Fourteen SNPs were validated in 960 women with severe PE and 705 non-PE controls from the FINNPEC cohort (including all of the FINNPEC participants included in the initial exploratory Sequenom genotyping). These data were combined with sequenced genotypes of *C3* in the initial phase and replication phase for the single SNP association analysis. The study subjects are described in **Table 1**.

Complement Genotyping

A custom SNP genotyping was performed using Sequenom's MassARRAY MALDI-TOF Mass Spectrometry Compact platform and iPLEX Gold chemistry (Sequenom Inc., San Diego, CA, USA) with standard protocols as described elsewhere (27). Briefly, 18 genes coding for components of the complement system were chosen for genotyping and for each gene, SNPs with assumed relevance were selected based on published data on protein function, activity, or disease association (Table S2 in Supplementary Material). Furthermore, we included potentially functional nonsense and missense SNPs, and finally, we also included some intronic, promoter or 3′ end SNPs as markers of association. A total of 98 SNPs were assayed in four multiplexes of 15–34 markers each. We focused on SNPs with minor allele frequencies >0.05 in European populations based on the HapMap data (28). Genotypes were analyzed using Sequenom's MassARRAY Typer version 4.0 software.

Microsatellite Analysis

The size of the $(CA)_n$ repeat polymorphism GF100472 in the promoter area of the *C3* gene was determined by fragment analysis. The repeat area was amplified by PCR using FAM-labeled forward primer and non-labeled reverse primer (Table S3 in Supplementary Material) in PCR conditions of an initial denaturation at 95°C for 10 min, followed by 32 amplification cycles of 95°C for 30 s, 67°C for 30 s, and 72°C for 50 s and final extension at 72°C for 7 min. The sizes of the amplified fragments were determined using an automated capillary sequencer ABI3730xl DNA Analyzer (Applied Biosystems). GeneScan 500 LIZ Size Standard (Applied Biosystems) was used to size the fragment data. The

FIGURE 2 | The haploblock structure within *C3* determined by Sanger sequencing and replication studies. The intensifying gradient of red represents the increasing relative linkage disequilibrium between two variants. The associating Block 2 is identified by the black triangle in the middle of the gene and had a multiallelic D′ score of 0.623. The single nucleotide polymorphisms (SNPs) that were assessed in each replication stage are identified by the respective color square that correspond to colors in the material panel in **Figure 1**. The independently associating SNPs are indicated by stars. All SNPs in this image are listed in Table S4 in Supplementary Material.

number of CA repeats was determined with Gene Mapper v4.0 software (Applied Biosystems) and the repeat alleles were classified as short length ($CA_{8–10}$), medium length ($CA_{11–12}$), or long (CA_{15}) for the purpose of analysis.

C3 Sequencing

The exonic areas of the *C3* gene including flanking intronic regions and potential splice sites and 650 kb PROM were sequenced using standard Sanger sequencing with primers detailed in S3. Amplitaq Gold (Applied Biosystems) enzyme was used in the reactions. Samples were purified from excess primers by digestion using 2.5 U of Shrimp Alkaline Phosphatase USB and 5 U of Exonuclease I (New England Biolabs) at 37°C for 60 min, followed by inactivation of 15 min at 80°C. Purified samples were prepared for sequencing using the BigDye 3.1 terminator (Applied Biosystems) as instructed by the manufacturer. The sequencing reaction was as follows: initiating step of 96°C for 1 min, 25 cycles of 96°C for 10 s, 53°C for 5 s, 60°C for 4 min. Sequence samples were purified with the Millipore Multiscreen plates (Millipore, USA) with Sephadex G-50 Superfine Sepharose (Amersham Biosciences, Sweden). Electrophoresis was performed with an ABI 3730 DNA Analyzer (Applied Biosystems) and base calling using the Sequence Analysis 5.2 software (Applied Biosystems). Initial analysis was carried out in Variant Reporter 1.0 software (Applied Biosystems) and the reported results were checked by Sequencher 4.1.4 software (Gene Codes, USA).

Replication by Sequenom Genotyping

Fourteen SNPs covering the length of the gene (indicated by red rectangles in **Figure 2**) were included in a single Sequenom iPLEX. The purpose of the Sequenom replication was to increase the sample size to gain reliability of analyses. The assay design and the genotyping were performed with Sequenom MassArray system at the FIMM Technology Centre, University of Helsinki. The Technology Centre performed routine quality control steps to ensure high quality of the genotyping.

Quality Control

Genotyping results from all methods were tested for deviations from the Hardy–Weinberg equilibrium ($p < 0.05$), and none were discovered in the controls. Data on three individuals were removed due to unresolved discrepancy between the sequenced and the genotyped results and data from 11 individuals were removed due to >10% failed genotyping by the Sequenom (Table S1 in Supplementary Material).

In Silico Analysis of Functionality

Ensemble Variant Effect Predictor and Human Splicing Finder 3.0 online softwares were used to assess the consequences of the five intronic SNPs of interest (29). RNAsnp by Center for non-coding RNA in Technology and Health (RTH) was the final online software that was applied to detect possible local RNA secondary structure changes, which might be introduced by exonic SNPs and could lead to changes in posttranscriptional processes of an otherwise functional gene (30, 31). Mode 2 of the program was used, as it is applicable for large mRNAs (>1,000 nucleotides). Following the suggested limits, a p-value <0.2 was considered indicative of an SNP induced change in secondary structure.

The Relative Extended Haplotype Homozygosity

To predict selection pressure toward the discovered haplotype and pinpoint its structure, we completed a REHH analysis using the associating rs2287845 as a focal SNP (32). REHH was conducted in R following the developer's instructions to reveal the evolutionary selection pressures underlying the haplotype structure. All Sanger sequenced individuals (initial cohort and replication cohort $N = 213$) were used for REHH and missing genotypes were imputed using fastPHASE software (33, 34).

Statistical Analyses

The results of the complement SNP genotyping were analyzed in PLINK (35). The *C3* sequencing results were analyzed for association in gPLINK and PLINK. Single SNP association to disease was evaluated by Fisher's Exact test and results were confirmed in Haploview (36). Haplotype analysis was conducted with the Haploview software (36). Association analysis of individual SNPs and the discovered haplotype blocks was done using χ^2 test in the Haploview program.

RESULTS

SNP Genotypes of Complement Genes

To analyze potential associations between PE and complement genes, we genotyped selected SNPs in 17 genes coding components of the complement system. No differences were observed in 64/72 SNPs between 259 PE women and 426 non-PE controls (data not shown). Out of the remaining eight SNPs, three associated to *C3* in genotypic model analysis and in *C3*, rs2230204, and rs2230205 were associated after permutation. rs2230204 in the proximity of *C3* exon 14 was most likely to have an independent effect (likelihood ratio test: $\chi^2 = 5.1$, df = 1, $p = 0.024$).

C3 Sequences

In the *C3* promoter, exons, and flanking introns, a total of 43 SNPs were identified in severe PE women, non-PE controls, or both (**Figure 2**). rs200046246 is located in a predicted transcription factor-binding site. It is a missense SNP that causes an amino acid change K779R. It was predicted by SIFT and POLYPHEN2 to be well tolerated and benign, apparently, because there is no change in the charge of the amino acid (both lysine/K and arginine/R are basic residues). Other variants were either synonymous or intronic. Six SNPs were independently associated to severe PE. rs190390034 (−39 from exon 2) had the strongest association with a predisposing effect with minor allele T [$\chi^2 = 7.72$, p-value = 0.005; OR = 7.627 (95% CI 1.442, 40.350)] (**Figure 2**). While we had appropriate power to assess three of six SNPs with single SNP association: rs2287845 [minor allele frequency (MAF) = 0.426, $p = 0.038$, OR = 1.158, 95% CI = 1.009, 1.331], rs366510 (MAF = 0.426, $p = 0.039$, OR = 1.158, 95% CI = 1.008, 1.330), and rs2287848 (MAF = 0.426, $p = 0.041$, OR = 1.155, 95% CI = 1.006, 1.327), the associations of the remaining three SNPs are only suggestive (**Table 2**).

TABLE 2 | Six non-coding single nucleotide polymorphism (SNP) have independent allelic association to severe preeclampsia.

Genomic position (Build 38)	SNP	Minor allele frequency in cases/controls	*N*	χ^2	*p*-Value	Odds ratio (CI95)
19:6686493	rs2241391	0.023/0.014	1,694	4.071	0.044	1.73 (1.009, 2.966)
19:6696331	rs2287848	0.441/0.405	1,693	4.163	0.041	1.155 (1.006, 1.327)
19:6696586	rs2287845	0.441/0.405	1,693	4.328	0.038	1.158 (1.009, 1.331)
19:6697818	rs366510	0.441/0.405	1,693	4.283	0.039	1.158 (1.008, 1.330)
19:6719442	rs190390034	0.078/0.011	123	7.72	0.005	7.627 (1.442, 40.35)
19:6720961	rs183805948	0.031/0	123	5.734	0.017	NA

CI95, 95% confidence intervals; NA, not available (the variant was not observed in controls).

TABLE 3 | The region covered by haploblock 2 had two haplotypes of 16 SNPs with suggestive association to preeclampsia.

Haploblock 2	Frequency cases/controls	Proposed effect	χ^2	*p*-Value	Odds ratio (*t*, Wald test)
CCCCCCCGC**T**CGACGC	0.274/0.366	Protective	4.047	0.044	0.628 (6.08)
CTTTTCTGG**C**CGCGAC	0.182/0.094	Predisposing	6.511	0.011	2.110 (4.72)

*The focal SNP rs2287845 in relative extended haplotype homozygosity analysis (**Figure 4**) is indicated in bold.*

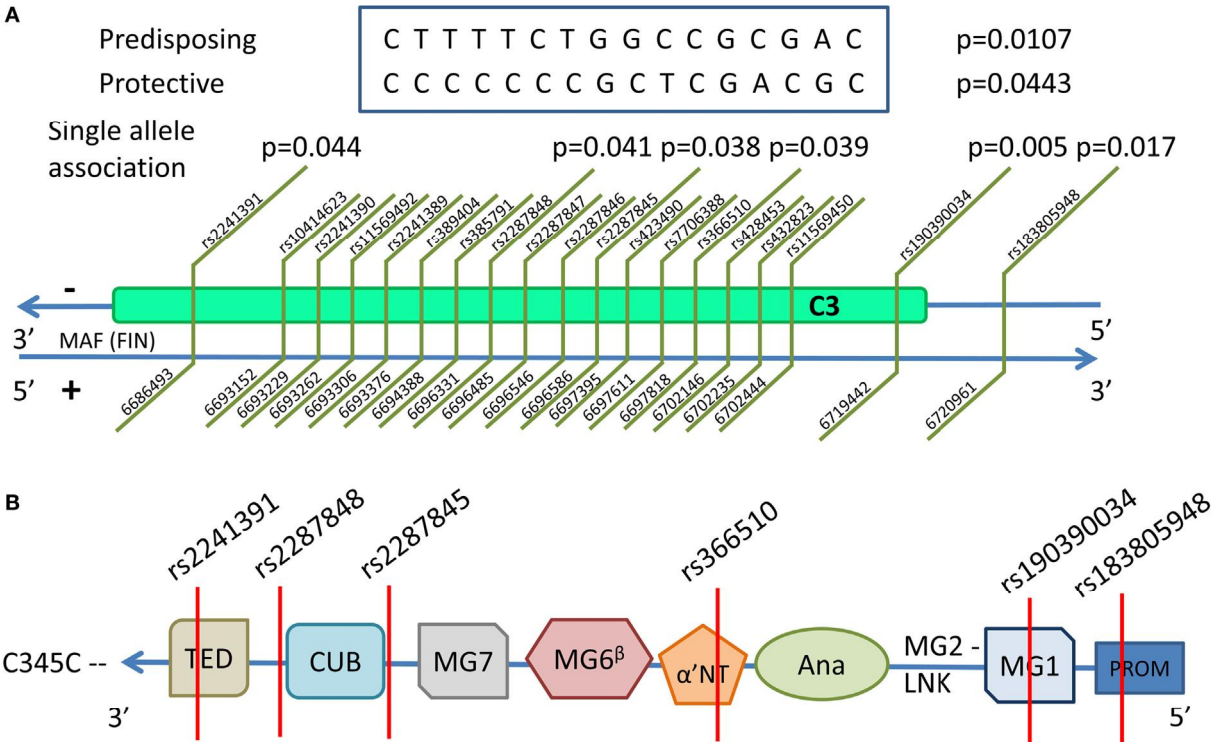

FIGURE 3 | *C3* is located in chromosome 19 in the reverse strand. The discovered single nucleotide polymorphism (SNP) associations, their positions, and associating haploblocks are depicted in **(A)**. The associating SNPs and their locations in the domains of the functional C3 are shown in the schematic structure in **(B)**. All associating SNPs are located in introns with the exception of rs183805948, which is located in the PROM. The domains are TED—thioester-containing domain (C3d); CUB—complement C1r/C1s, Uegf, Bmp1; MG1–7—macroglobulin domains 1–7; α'NT—N-terminal region of the cleaved α-chain in the linker domain; Ana—anaphylatoxic region (C3a); LNK—β-strand of the linker domain; PROM—promoter region.

A set of alleles spanning from 5′ intron proximal to exon 18 to the 5′ intron proximal to exon 25 and consisting of 16 SNPs was found to be associated with severe PE. One haplotype showed an association in a protective (frequency in severe PE women and non-PE controls 0.275 and 0.366, respectively, $p = 0.044$), and another one in a predisposing manner (frequency in severe PE women and non-PE controls = 0.182 and 0.094, respectively, $p = 0.011$) (**Table 3**). Mapping the 16 SNPs contributing to the haplotype along the *C3* gene showed that the tagging SNP rs2287845 is located 7bp in 3′ direction of exon 22, which corresponds to the alpha-chain of the gene product at the edge of MG7 and directly before (5′) the CUB domain (**Figure 3**). The

proposed haplotype spans across the middle of the gene from the 3′intron of exon 18 situated at the 5′end of the ANA domain right before the α′NT domain and at 5′direction reaching past the CUB and into the TED domain. Three of the SNPs in the haplotype (rs406514, rs11569450, and rs432823) are located in the introns flanking exon 18, which codes for the α′NT domain. The regulatory microsatellite region was not associated with severe PE.

In Silico Functional Analysis

Human Splicing Finder 3.0 found splicing motives to be influenced by four of the associating *C3* SNPs: rs190390034, rs2287848, rs366510, and rs2241391. rs366510, rs2241391, and rs190390034 were predicted to create an exonic splicing enhancer (ESE) site within an intron, whereas rs2287848 was found to create a novel ESE site as well as to cause an alteration of an existing ESE site. In RNAsnp, rs190390034 had a *p*-value of 0.029 indicating a disruption in local RNA folding and rs2241391 had a *p*-value of 0.169 indicating a likely disruption in local RNA folding. The associating SNPs within the haploblock region (rs2287845, rs366510, and rs2287848) had RNAsnp *p*-values >0.2 indicating no significant structural change caused by these variants.

The Relative Extended Haplotype Homozygosity

The tagging SNP rs2287845 was associated with an extended haplotype to the 5′ direction on the *C3* gene suggesting that this structure with tight linkage disequilibrium in the middle of the gene results from a positive selection pressure (**Figure 4**).

DISCUSSION

In this study, we have described genetic variants of the key complement component C3 in women with severe PE and control women with non-PE pregnancies. We identified a 16-nucleotide haplotype signature in the highly conserved middle region of the maternal *C3* gene with three associating SNPs (rs2287845, rs366510, and rs2287848) that could influence susceptibility to severe PE.

The three *C3* SNPs that associate with severe PE are located in the area of most intense linkage disequilibrium in the middle of the associating haploblock. The discovery of predisposing SNPs within the haploblock structure supports a possible functional role for the haploblock-encoded gene product. Furthermore, rs190390034 is an intronic variant 38 bp 5′ downstream of exon 2 that is in tight linkage disequilibrium with the haploblock structure in the middle of the gene. rs190390034 has a perfect D′ score of 100 for the tagging SNP rs2287845 (**Figure 2**). Among 43 observed SNPs, rs190390034 had the best allelic association with severe PE. While suggestive due to insufficient sample size, the independent association of rs190390034 with severe PE supports the role of the haploblock in severe PE. With the available genotypes for the remaining two of the six associating SNPs within *C3* we were underpowered due to insufficient sample size (rs190390034) and rarity of the variants (rs183805948 and rs2241391). Therefore, the latter results should be interpreted with caution.

In support of the causality of changes in the haploblock 2 area of the *C3* gene, two of the three associating SNPs within the haploblock described in our study have been implicated in prior studies in other phenotypes. rs2287845*C has been shown to be significantly associated with overall survival and prognosis in patients with early stage non-small cell lung cancer after surgical resection (37). rs366510 is a probable splicing variant that has been linked to asthma and related phenotypes in two independent studies (38, 39). None of these studies looked for a haplotype association within *C3*. Intronic variants causing *C3* splicing mutations have been described in patients with C3 deficiency due to exon deletion from the C3 mRNA (40, 41). The current study is according to our knowledge the first one to show a disease association in C3 with a haplotype-based mechanism instead of the conventional single SNP association. Indeed, it has become apparent that while mutations leading to changes in amino acid sequences are readily detected, more subtle changes in gene regulatory elements are most likely accountable for much of the phenotypic variation we observe in complex diseases (42, 43). Such regulatory features remain cryptic in analyses at the translational and posttranslational level.

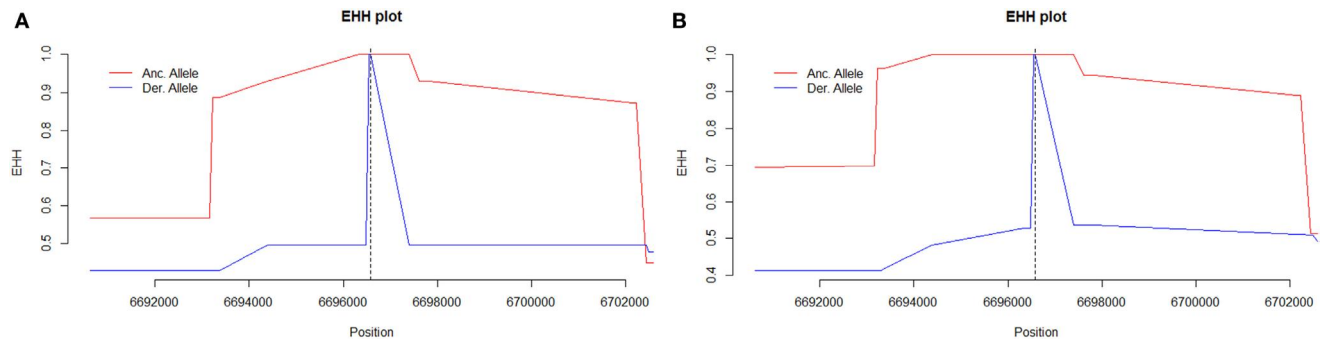

FIGURE 4 | The extended haplotype homozygosity (EHH) diagram of (A) women with severe preeclampsia (PE) and (B) non-PE controls using rs2287845 as a focal single nucleotide polymorphism (SNP) representative of the haploblock structure. The ancestral allele curve (in red) is close to 1 for a long stretch in non-PE controls particularly in the 5′ upstream direction indicating that the haplotype surrounding the focal SNP (indicated by severed vertical line) is under selection in this cohort. Major allele (ancestral, in red) C, minor allele (derived, in blue) T.

In other genes, intronic disease associations with causative splicing defects have been described (44, 45). In the field of reproductive immunology in a set-up similar to our study, an intronic haplotype was found to result in IL-10 secretion changes in women with idiopathic recurrent miscarriage (46). Furthermore, it was recently shown that mechanical nucleosome binding occurs even on top of genes (47). Therefore, it is possible that non-coding SNPs may have an important regulatory role, e.g., by influencing DNA folding.

Complement C3 plays a central role in a successful pregnancy. Inappropriate complement activation may play a role in the initial stages of PE pregnancies contributing to inadequate placentation or placental dysfunction. The anecdotal reports of success of eculizumab in the treatment of a full-blown disease indicate that the complement system is also involved in the later stages of the disease possibly by generating inflammation or tissue damage (22, 48). Problems may emerge if disturbances in the removal of ischemic or injured placental components by complement and phagocytes occur (49). Lack of functioning C3 in mice led to fewer pregnancies and to a higher fetal reabsorption rate, while fetal and placental weights were lower (50). On the other hand, C3-mediated over-activation of the complement system was shown to induce hypertension following placental ischemia in rats (51). Furthermore, complement activation at the feto-maternal interface of Crry$^{-/-}$ mice that lack a key complement regulator was shown to cause fetal loss. The embryos were rescued when Crry$^{-/-}$ mice were bred to C3$^{-/-}$ mice (52). These observations underline the importance of balanced activation and regulation of the complement system for a healthy pregnancy.

Because C3 activation by the C3 convertases requires extensive conformational changes and translocation of the CUB/TED and α′NT domains (17), protein changes caused by variants in the middle of the *C3* gene may hinder binding of factor B to C3b causing the C3 convertase to function inefficiently (**Figure 3**). If the haplotype described here has an effect on C3 function as suggested by its critical location, it is possible that C3 activation in the individuals with the protective haplotype is properly regulated. Thereby, the extravillous trophoblasts (EVTs) invading the maternal tissue during placentation would not encounter a vigorous complement attack. Thus, they could successfully remodel the uterine spiral arteries resulting in a non-PE pregnancy with a healthy blood flow and placental development (9). Concurrently, the predisposing haplotype may result in an increased level of complement activation as indicated by increased factor B (Bb) levels early in the pregnancy. Complement attack could compromise the EVT invasion and consequently the placental function resulting in an increased occurrence of severe PE (11, 53).

C3 promoter activity is dependent on the dinucleotide repeat polymorphism GF100472 such that the longer the CA repeat region, the lower the transcriptional activity of *C3* (54). A shorter repeat has shown protective effect against mesial temporal lobe epilepsies and febrile seizures. However, in the present study, we did not find any indication of association of the CA-repeat to severe PE in a small patient cohort.

The functional polymorphism rs2230199 in *C3* is known as the slow/fast mutation that influences C3 protein mobility in electrophoresis gels. C3F has been described as predisposing to PE (55), but we did not find any association of C3F with severe PE. Our result concurs with an early study of C3 allotypes that did not find association of C3F with PE (56).

The REHH analysis shows that the haploblock structure in the middle of *C3* is tightest for the ancestral rs2287845 allele in non-PE controls suggesting that the structure results from a positive evolutionary selection pressure (**Figure 4B**). A similar pattern is observed for the ancestral allele of rs2287845 in PE women. However, in severe PE, the haploblock structure disintegrates noticeably sooner than in controls, indicating a loosened force of active selection (**Figure 4A**). It would follow that due to a possible regulatory feature caused by seemingly benign variants that have been introduced into the middle of *C3*, the risk for severe PE increases, while haplotypes with the ancestral genotype are protective from severe PE and, accordingly, under stronger positive natural selection.

The heterogeneity of PE is reflected in the comparison of our results to another recent study. Wu et al. found that rs698090 in *MASP1* is associated to late-onset but not to early-onset PE, and nominally to severe PE in a Chinese population (57). In our initial genotyping, we did not find allelic or genotypic association of rs608090 to severe PE. It is possible that different complement pathways contribute to early-onset PE and late-onset PE and the mechanism of these varying associations merit more studies.

Targeting gene regulatory effects may provide new opportunities for PE risk assessment and diagnosis, maybe even future drug development (58). The reported results reveal significant differences between PE and healthy pregnant women but the roles of individual SNPs should be considered suggestive and treated with caution. With further studies to confirm our findings, assessing *C3* genetic polymorphisms may be developed as a tool to find patients with the highest risk of severe PE.

ETHICS STATEMENT

All subjects provided a written informed consent in accordance with the Declaration of Helsinki. Study protocols were approved by the local Ethical Committees, specifically, for the FINNPEC study, ethical approval has been obtained from the Coordinating Ethics Committee, Hospital District of Helsinki and Uusimaa, for the Finnish population based pre-eclampsia cohort was approved by the ethics committee of the Finnish Red Cross Blood Service and by the Ministry of Social Affairs and Health and for Southern Finland pre-eclampsia study was approved by the Ethics Committee of the Department of Obstetrics and Gynaecology at Helsinki University Central Hospital.

AUTHOR CONTRIBUTIONS

AIL designed the study with SM and HL. AIL and TK designed part of the primers for sequencing, AIL supervised laboratory work, sequenced all samples from patients and some controls, read the results, conducted association analyses of the *C3* sequencing project, performed *in silico* analyses, and drafted the manuscript. TK conducted laboratory work for the regulatory regions branch of the study, analyzed these data, and participated in main data

analysis. VH designed the complement genotyping Sequenom chip. PO analyzed the genotyping data. LK performed and interpreted the REHH analysis with AIL and PS. PV described patient cohorts. HL, SH, EK, JK, KK, and AP form the board of the FINNPEC cohort and are responsible for the clinical data and biological samples used in this study. LH provided samples and is with HL responsible for the clinical data of the Finnish population-based preeclampsia cohort. HL provided the samples and clinical data of The Southern Finland preeclampsia cohort. All the authors collaborated in drafting the manuscript and accepted the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00589/full#supplementary-material.

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Protective Low-Frequency Variants for Preeclampsia in the Fms Related Tyrosine Kinase 1 Gene in the Finnish Population

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Abstract—Preeclampsia is a common pregnancy-specific vascular disorder characterized by new-onset hypertension and proteinuria during the second half of pregnancy. Predisposition to preeclampsia is in part heritable. It is associated with an increased risk of cardiovascular disease later in life. We have sequenced 124 candidate genes implicated in preeclampsia to pinpoint genetic variants contributing to predisposition to or protection from preeclampsia. First, targeted exomic sequencing was performed in 500 preeclamptic women and 190 controls from the FINNPEC cohort (Finnish Genetics of Preeclampsia Consortium). Then 122 women with a history of preeclampsia and 1905 parous women with no such history from the National FINRISK Study (a large Finnish population survey on risk factors of chronic, noncommunicable diseases) were included in the analyses. We tested 146 rare and low-frequency variants and found an excess (observed 13 versus expected 7.3) nominally associated with preeclampsia ($P<0.05$). The most significantly associated sequence variants were protective variants rs35832528 (E982A; $P=2.49E-4$; odds ratio=0.387) and rs141440705 (R54S; $P=0.003$; odds ratio=0.442) in Fms related tyrosine kinase 1. These variants are enriched in the Finnish population with minor allele frequencies 0.026 and 0.017, respectively. They may also be associated with a lower risk of heart failure in 11 257 FINRISK women. This study provides the first evidence of maternal protective genetic variants in preeclampsia. (***Hypertension*****. 2017;70:365-371. DOI: 10.1161/HYPERTENSIONAHA.117.09406.) • Online Data Supplement**

Key Words: cardiovascular diseases ■ Finland ■ heart failure ■ preeclampsia ■ proteinuria

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Preeclampsia is a common vascular disorder that affects 3% of pregnant women.[1] Worldwide, it annually accounts for ≈50 000 maternal and 900 000 perinatal deaths.[2,3] The clinical characteristics are diverse, and the course of the disease is unpredictable. Both a preeclamptic mother and a child born from a preeclamptic pregnancy are at higher risk for later-life cardiovascular diseases and type 2 diabetes mellitus.[4]

Angiogenesis is tightly involved in the pathophysiology of preeclampsia.[5] A high ratio of sFLT1 (soluble Fms related tyrosine kinase 1) to placental growth factor is among the most promising biomarkers for predicting the onset of the disease.[6] Several susceptibility loci for preeclampsia have been identified in genome-wide linkage studies.[7,8] However, linkage and candidate gene studies have been plagued with poor reproducibility.

The population of Finland is genetically unique in Europe.[9] The repeated bottleneck events that caused strong founder effects and geographic isolation over centuries have resulted in the enrichment of variants that are rare or absent in other populations.[10] These features provide an opportunity

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to overcome many of the obstacles in studying rare enriched variants that contribute to the risk of complex diseases.[11]

We designed a targeted gene sequencing protocol to screen the coding and splicing areas of genes of interest within angiogenic and vascular pathways and other putative candidate genes in a sizeable cohort of preeclampsia cases and controls from Finland. Here, we explore the potential causal role of variation in candidate genes in preeclampsia.

Methods

Diagnostic Criteria and Patient Cohorts

The study design is outlined in Figure. At stage 1, we studied 500 nonobese (body mass index <30 kg/m^2) women with preeclamptic pregnancies and 190 nonpreeclamptic controls that were matched geographically, in age, and in body mass index from the FINNPEC cohort (Finnish Genetics of Preeclampsia Consortium), a case–control cohort recruited from the 5 Finnish University Hospitals.[12] Nulliparous or multiparous women with a singleton pregnancy were eligible for the study. Preeclampsia was defined as hypertension and proteinuria occurring after 20 week's gestation. Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg after 20 weeks of gestation. Proteinuria was defined as the urinary excretion of ≥0.3 g protein in a 24-hour specimen, or 0.3 g/L, or 2 ≥1+ readings on dipstick in a random urine determination with no evidence of urinary tract infection. All diagnoses were independently verified by a research nurse and a physician. Seven preeclamptics and 1 control were excluded because of a failure in genotyping, and further 9 cases were excluded because of non-Finnish ethnicity and ovum donation pregnancy. The characteristics of the study participants are presented in Table S1 in the online-only Data Supplement. All women provided a written informed consent, and the FINNPEC study protocol was approved by the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa.

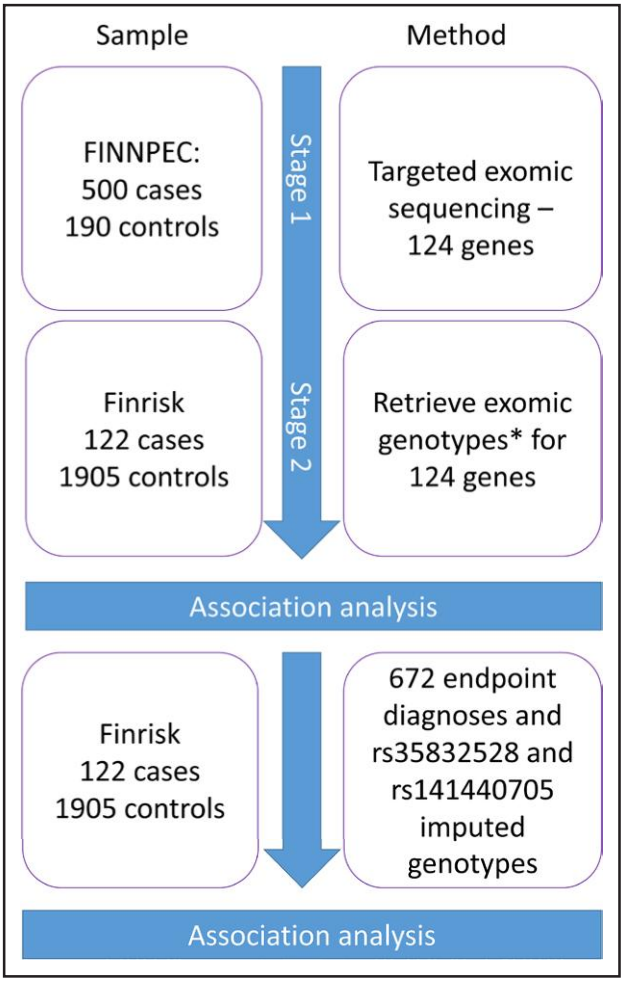

Figure. Methods and samples of study design. *Genotyping performed by Agilent 1.1 refseq (60 cases and 4 controls), Illumina coding v1 (162 cases and 10 controls), and Nimblegen SeqCap EZ VCRome (1682 cases and 109 controls) platforms. FINNPEC indicates Finnish Genetics of Preeclampsia Consortium.

At stage 2, we included in the analyses whole-exome sequencing from an additional 122 women with a history of preeclampsia and 1905 parous women with no such history from the national FINRISK study cohort (FINRISK license 8/2016).[13] For identifying preeclampsia and eclampsia cases in the FINRISK study cohort, we used following Finnish *International Classification of Disease* (*ICD*) codes in the comprehensive National Hospital Discharge Register covering years 1992 to 2007: *ICD-10* (in use since 1996): O14.0, O14.1, O14.9, O15.0, O15.1, O15.2, O15.9; *ICD-9* (in use from 1987 to 1996): 6424 to 6426, 6427A; and *ICD-8* (in use from 1968 to 1986): 637.03, 637.04, 637.09, 637.10, 637.99. Controls from FINRISK were all women who had given birth at least once and did not have any of these diagnoses recorded. In the final association analyses, we included genotypes of FINNPEC and FINRISK which combined totaled 609 cases and 2092 controls.

All polymorphic sites in the stage 1–targeted sequencing were queried from the population cohort exomes. All of the National FINRISK study methodology and ethical approvals are available online: https://www.thl.fi/en/web/thlfi-en/research-and-expertwork/population-studies/the-national-finrisk-study.

Preparation of Genomic DNA

Genomic DNA was extracted from whole blood using the NucleoSpin Blood XL DNA extraction kit (Macherey-Nagel GmbH & Co.) or the Chemagic Magnetic Separation Module I–machine (Chemagen) and subsequently stored at −20°C.

Targeted Sequencing and Capture Enrichment

Genes implicated in human biomarker studies were chosen as targeted candidate genes as were their signaling partners and proteases that alter their function and levels. Specific single nucleotide polymorphisms (SNPs) and any neighboring or associated gene that have been enriched in preeclamptic women based on meta-analysis, genome-wide association studies, or functional studies were included. This list of existing candidate genes was based on review of literature in January 2013, and it included genes involved in angiogenesis, such as *FLT1*, and pregnancy, such as pregnancy-zone protein, among other relevant functional pathways. The studied genes are listed in Table S2.

Libraries from genomic DNA were prepared in-house (Washington University School of Medicine).[14] Enzymes were purchased from Enzymatics (Beverly, MA). Briefly, the ends of sheared genomic DNA fragments were repaired by treatment with T4 DNA polymerase and T4 DNA polynucleotide kinase, which phosphorylates the 5′ hydroxyl group. An adenosine was then added to the 3′ position at each end of the DNA fragment with Taq Polymerase. Illumina adapters with an overhanging T were ligated onto the DNA fragment followed by bead-based size selection procedure to remove adapter dimers and fragments below the desired size. A unique index sequence was added by polymerase chain reaction by targeting the 2 ligated universal adapters on each fragment end.

Sequence capture by hybridization was performed according to the manufacturer's recommendations with modifications (Roche SeqCap Hybridization and Wash Kit No. 05634261001). We used longer blocking oligos containing an additional 7 bp inosine segment for promiscuous pairing with different index sequences. After hybridization, captured DNA was washed and eluted according to the manufacturer's instructions.

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Sequencing and Analysis

Sequencing was performed on an Illumina HiSeq 2000 at the Washington University Genome Technology Access Center using 2×101 bp, 2×135 bp, and 2×150 bp reads. We aligned sequencing to GRCh37 using bwa aln (v0.6.1-r104) and genotyped the samples using the Genome Analysis ToolKit (v2.5.2-gf57256b) Unified Genotyper.[15–17]

Statistical Analyses

Quality control before meta-analysis included removal of singleton and monomorphic variants, removal of sites with >10% missing data in the targeted sequencing, or a significant departure from Hardy–Weinberg equilibrium in controls (P<0.001). The combined data set was analyzed using Fisher exact test that served as our primary association test. The analysis was divided into 2 classes: low frequency and rare variants (minor allele frequency [MAF] <10%) or common variants (MAF>10%). After quality control, 622 variants (443 variants with MAF<10% and 179 variants with MAF>10%) were advanced to the combined primary analysis. Of these, 201 were in putatively functional categories (missense, nonsense, or splice region variants) and 421 were in likely benign categories (synonymous, intronic, and intergenic).

The 2 SNPs with strongest associations were further compared with 672 predefined epidemiological diagnoses derived from national healthcare registries using imputed genotypes of 11 257 FINRISK women. Genotypes were imputed using a combined panel of Finnish whole-genome sequences and 1000 Genomes phase I reference with a high-imputation confidence (info metric >0.97).[18,19] Associations were calculated using logistic regression (SNPTEST v2.5.2, EM algorithm) and then further confirmed with Fisher exact test given the rarity of variants and several diagnoses. Because of the explorative nature of this part of the analysis, no multiple testing corrections to estimates were performed. Heart failure was identified using codes I50, 4289X, and 42700 in Finnish *ICD-10*, *ICD-9*, and *ICD-8*, respectively.

Data were analyzed using PlinkSeq, Plink,[20] and R. Kaviar,[21] and VEP Build 37 was used in additional annotations.[22] Loss of function (LoF) analyses were conducted for each gene with associating variants in silico by the LoF tool (https://github.com/ensembl-variation/VEP_plugins/blob/master/LoFtool.pm). The following annotations were calculated: LoF score <0.2 is probably damaging, LoF score 0.2 to 0.7 is possibly damaging, LoF score <0.7 is benign. In addition to appropriate statistical probability tests, odds ratios (OR) with 95% confidence intervals were calculated for all variants.

Results

Results of the association analyses are listed in Table. For these studies, genotypes from stage 1 (FINNPEC) were combined with a population cohort in stage 2 (FINRISK) for joint analysis. Forty-one variants demonstrated nominal association (P<0.05; 34 uncommon and 7 common). The tail of the P value distribution of benign variants was as expected (0 results with P<0.001; expected ≤1 and 2 results with P<0.01; expected ≤4.2), suggesting that the overall study design and quality control were successful. Among the 201 putatively functional variants, one had P<0.001 (α=0.05) compared with the 0 of 421 observed among benign variants. The lack of inflated P values points out that confounders, such as stratification, are not causing false positives.

Focusing on 146 putatively functional (missense or truncating) rare and low-frequency variants, we observed an excess (observed 13, expected 7.3) of nominally associated variants (P<0.05). The strongest associations observed were in *FLT1*, with 2 missense variants associated to preeclampsia: NM_002019.4:p.E982A (rs35832528; P=2.5E-4; OR=0.387, withstands correction for multiple testing for the 146 rare and low-frequency coding variants) and NM_001159920.1:p.R54S (rs141440705; P=0.0027; OR=0.44). These variants are enriched in the Finnish population with MAFs being 0.026 (1000 Genomes MAF=0.0014) and 0.017 (1000 Genomes MAF=0.0008), respectively (www.sisuproject.fi).

E982A and R54S were examined across registry-derived disease end points. Both of the assessed SNPs protected from heart failure (Fisher exact P=0.007 for both, E982A: OR=0.368; 95% confidence interval=0.164–0.830; cases/controls=301/10 956; minor allele [G] count in cases/controls=6/593; R54S: OR=0.340; 95% confidence interval=0.140–0.826; cases/controls=297/10 960; minor allele [G] count in cases/controls=5/543).

Discussion

We discovered low-frequency protective genetic variants in *FLT1* that contributed to lower preeclampsia risk. We also found associating genetic variants in known candidate genes using a targeted sequencing approach. These *FLT1* variants may also be associated with lower risk of heart failure.

FLT1 codes for VEGFR1 (vascular endothelial growth factor receptor 1). It consists of 7 immunoglobulin-like domains in an extracellular ligand-binding region, a transmembrane segment, and a cytoplasmic region containing a tyrosine kinase domain.[23] VEGFR1 is essential for survival by negatively regulating the levels of endogenous vascular endothelial growth factor. Internalization and signaling of functional VEGFRs will enhance angiogenic growth of blood vessels.[24] Sustaining angiogenesis is necessary for circulation, and anti-angiogenic treatment causes cardiovascular morbidity.[25] There are multiple isoforms of VEGFR1 of which the soluble forms have been implicated in preeclampsia. The soluble forms of VEGFR1 only contain the extracellular parts of the protein encoded by the first 13 of 30 exons. In preeclampsia, an excess of VEGFR1 of placental origin has been recorded.[26] Furthermore, after healthy endothelium is restored, elevated levels of soluble VEGFR1 are observed in women with a history of preeclampsia.[27]

Increased levels of sFLT1/sVEGFR1 have also been indicated in peripartum cardiomyopathy where high levels of sFLT1 correlate with the symptoms' severity.[28] Similarly, heart failure after myocardial infarction independent of pregnancy is reflected in extreme levels of sFLT1.[29] Rhee et al report extreme sFLT1 levels in at least the 95th percentile in a cases of heart failure during pregnancy.[30] Although further research is required to assess the effect of underlying risk factor profile in preeclamptic women's increased risk of heart failure in later life, it was recently shown in a meta-analysis of results from 7 studies that 3.6-fold increase in risk of heart failure is associated with preeclampsia, particularly during the time period of 1 to 10 years after preeclampsia.[31] Preeclampsia increases the risk of peripartum cardiomyopathy, and it has been suggested that sFLT1 may be toxic to the heart. Also the driver of the cardiac dysfunction in susceptible preeclamptics is likely mediated by antiangiogenic factors.[32] Our results indicate that *FLT1* variants that protect from preeclampsia may also protect from heart failure, thereby adding to the growing body of evidence that imbalance of angiogenic factors may be the link between preeclampsia

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Table. The Observed Variants With Significant Associations to Preeclampsia

RSID Number	Gene Name	*P* Value*	Odds Ratio (95% Confidence Interval)	MAF in Total Sample	Count With Minor Allele $n_{Cases}/n_{Controls}$ FINNPEC Minor Allele Count in Parentheses (Cases/Controls)	HWE	Consequence (Distance From Exon, Base Pairs)	LoF Tool
rs35832528	*FLT1*	2.49E-4	0.387 (0.205–0.678)	0.010	14/122 (7/6)	1	Missense variant, E982A	0.463
rs141440705	*FLT1*	0.003	0.442 (0.233–0.779)	0.010	14/106 (8/5)	1	Missense variant, R54S	0.463
rs61758484	*CORIN*	0.003	2.658 (1.320–5.261)	0.013	17/22 (16/1)	1	Noncoding transcript exon variant, E10K	0.201
rs34106916	*ANGPTL1*	0.006	3.186 (1.325–7.597)	0.008	12/13 (8/3)	1	Synonymous variant, Q103Q	0.658
rs61759670	*CORIN*	0.010	2.082 (1.147–3.693)	0.016	21/35 (18/4)	1	Missense variant, Y907T	0.201
rs80338240	*JAG1*	0.010	0.256 (0.051–0.806)	0.004	3/40 (2/4)	1	Intron variant (–11)	0.006
rs147998709	*GPR98*	0.011	13.757 (1.360–675.490)	0.003	4/1 (3/0)	0.004	Intron variant (+24)	0.977
rs2290843	*ADAM12*	0.011	0.726 (0.561–0.932)	0.071	83/383 (63/33)	1	Synonymous variant, T326T	0.320
rs61760500	*CORIN*	0.011	2.550 (1.179–5.382)	0.010	14/19 (11/3)	1.001	Intron variant (+13)	0.201
rs147942437	*ADAM28*	0.012	5.181 (1.226–24.998)	0.004	6/4 (5/0)	1.002	Missense variant, L449P	0.994
rs13406336	*ACVR1*	0.013	2.423 (1.129–5.062)	0.011	14/20 (13/2)	1.003	Missense variant, A15G	0.138
rs142436579	*ADAM28*	0.014	0.366 (0.129–0.852)	0.005	6/56 (4/3)	1.004	Missense variant, R219S	0.994
rs140437272	*INHBE*	0.014	0.477 (0.236–0.881)	0.013	12/85 (11/6)	1.005	Missense variant, P27L	0.786
rs201756397	*FLT4*	0.014	Inf (1.283–Inf)	0.001	3/0 (2/0)	1.006	Synonymous variant, E926E	0.023
rs80069610	*GPR98*	0.016	2.499 (1.122–5.411)	0.010	13/18 (11/2)	1.007	Synonymous variant, V1101V	0.977
rs139608664	*INHA*	0.018	5.716 (1.110–36.859)	0.003	5/3 (4/0)	1.008	Synonymous variant, S225R	0.046
rs4556933	*ACVR1C*	0.019	0.847 (0.735–0.976)	0.323	1157/381 (314/120)	0.113	Synonymous variant, F38F	0.076
rs41302834	*GPR98*	0.022	3.451 (1.031–11.557)	0.007	7/7 (7/2)	1.009	Missense variant, D1944N	0.977
rs3736061	*FLT4*	0.023	0.803 (0.665–0.973)	0.135	485/175 (143/39)	0.868	Synonymous variant, L252L	0.023
rs3741849	*PZP*	0.039	1.309 (1.004–1.696)	0.068	88/235 (71/21)	1	Synonymous variant, K563K, splice region variant	0.988
rs34307240	*LCT*	0.026	2.201 (1.038–4.515)	0.010	14/22 (12/2)	1.011	Missense variant, D106E	0.571
rs36032184	*INHA*	0.027	1.906 (1.058–3.346)	0.017	21/38 (20/3)	1.012	Synonymous variant, G109G	0.046
rs2228048	*TGFBR2*	0.029	0.672 (0.459–0.963)	0.030	38/191 (27/13)	0.450	Synonymous variant, N354N	0.060
rs138819536	*INHBA-AS1*	0.032	4.311 (0.926–21.758)	0.005	5/4 (4/1)	1	Missense variant, R229Q	0.043
rs1466360	*ADAM12*	0.033	1.152 (1.010–1.315)	0.414	1852/504 (404/152)	0.691	Intron variant (+30)	0.320
rs56133834	*TEK*	0.034	3.450 (0.921–12.934)	0.004	6/6 (6/0)	1	Synonymous variant, E986E	0.046
rs140593977	*TREX1*	0.034	3.448 (0.920–12.928)	0.004	6/6 (6/0)	1	Downstream gene variant (–44)	0.824
rs1466361	*ADAM12*	0.034	1.152 (1.010–1.316)	0.414	1810/501 (404/152)	0.691	Intron variant (–46)	0.320
rs148671842	*EHD3*	0.035	2.372 (0.992–5.459)	0.007	11/16 (8/2)	1	Synonymous variant, E188E	0.139

(*Continued*)

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Table. Continued

RSID Number	Gene Name	*P* Value*	Odds Ratio (95% Confidence Interval)	MAF in Total Sample	Count With Minor Allele $n_{Cases}/n_{Controls}$ FINNPEC Minor Allele Count in Parentheses (Cases/Controls)	HWE	Consequence (Distance From Exon, Base Pairs)	LoF Tool
rs115734907	*KDR*	0.035	0.695 (0.483–0.979)	0.029	42/205 (28/11)	1	Intron variant (+12)	0.196
rs1554286	*IL10*	0.037	1.203 (1.009–1.438)	0.153	761/191 (145/60)	0.231	Intron variant (+18)	0.538
rs2453040	*NOTCH2*	0.037	0.818 (0.676–0.992)	0.141	482/172 (144/45)	0.423	Intron variant (–45)	0.016
rs116951780	*LCT*	0.038	10.333 (0.829–541.218)	0.001	3/1 (2/0)	1	Synonymous variant, NMD transcript variant, A921A	0.571
rs138894008	*TEK*	0.038	10.333 (0.829–541.218)	0.002	3/1 (3/0)	1	Missense variant, R479H	0.046
rs148588802	*FLT1*	0.038	10.328 (0.829–540.960)	0.002	3/1 (3/0	1	Intron variant (+40)	0.463
rs200071734	*FLT4*	0.040	10.114 (0.811–529.805)	0.002	3/1 (3/0)	1	Missense variant, V157M	0.023
rs150123876	*ANGPT4*	0.042	0.348 (0.090–0.968)	0.007	4/39 (3/7)	1	Missense variant, R25H	0.773
rs368518386	*FLT4*	0.046	5.581 (0.799–61.819)	0.003	4/2 (4/0)	1	Intron variant (–43)	0.023
rs3736062	*FLT4*	0.046	1.547 (0.988–2.374)	0.024	33/74 (26/6)	1	Synonymous variant, Y531Y	0.023
rs7830	*NOS3*	0.049	0.877 (0.769–1.002)	0.434	1635/525 (411/172)	0.346	Intron variant (+11)	0.817
rs61763183	*FLT1*	0.050	1.653 (0.967–2.754)	0.020	24/50 (20/7)	1	Downstream gene variant (+73)	0.463

Counts of minor alleles are given for the combined data; 615 cases and 2094 controls, and per FINNPEC data set (in parentheses); 487 of 500 women with preeclampsia and 187 of 190 control women passed quality control. FINNPEC indicates Finnish Genetics of Preeclampsia Consortium; HWE, Hardy–Weinberg Equilibrium; LoF, loss of function; MAF, minor allele frequency; and RSID, identifying number.

*Nonadjusted *P* values.

and cardiovascular morbidity in later life in susceptible individuals. Novel therapeutic options for these individuals may include blocking sFLT1 production or neutralizing antibodies against angiogenic proteins.[33,34]

From the observed associating variants in *FLT1*, rs141440705 causing R54S is more likely of functional importance. This is because it results in a polar change (pos→neutral) within the immunoglobulin-like domain 1, a functional part of the protein.[35] Rs141440705 is located in the last nucleotide of an exon. Therefore, the variant may affect splicing, as well as the coded amino acid sequence.

Epidemiological studies with many of the protective associations from severe cardiovascular diseases are strongest for R54S, supporting the suggestion of functional significance. The other variant resulting in the amino acid substitution E982A is not included in the sFLT1 or the VEGFR1 isoform 2, which is the dominant isoform in the placenta. Even so, because VEGFR1 isoform 1 (canonical sequence) is expressed in vascular endothelium, E982A might have an important role in pregnancy in mediating decidual blood flow and in remodeling the spiral arteries.

We also found support for the role of several other candidate genes in preeclampsia. *Corin* has been previously indicated in preeclampsia with population-specific variants.[36] Stepanian et al[36] report similar ORs of ≈2.5 at 2 intronic SNPs, rs2271036 and rs2271037 to our finding at rs61759670 and rs61760500. Rs13406336 and rs4556933 in activin A receptor type 1C have been previously listed as a candidate SNPs although no significant association to preeclampsia was established in a Norwegian population.[37] Interestingly, rs7830 features in numerous studies that pinpoint the association of nitric oxide synthase 3 to a spectrum of multifactorial diseases. Most relevantly, a haplotype including rs7830 was found to protect women from pregnancy hypertension and preeclampsia.[38] Rs1554286 in interleukin 10 has been shown to belong to an intronic haplotype, which strongly predisposes women of Bahraini Arab population to idiopathic recurrent miscarriages.[39] Our findings support the hypothesis that common immunologic pathogenesis may be shared between recurrent miscarriages and preeclampsia.[40,41]

Perspectives

To our knowledge, this study provides the first evidence that maternal *FLT1* sequence variants associate with lower preeclampsia susceptibility. Further research is required to pinpoint the mechanism of protection from preeclampsia and heart failure and the effect of *FLT1* variants on gene transcription. Genetic associations may open new avenues of drug development once the functional consequences of our findings are further deciphered. Genetic protection from preeclampsia because of *FLT1* variants may also protect these women from heart failure in later life.

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Disclosures

None.

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Novelty and Significance

What Is New?

- We have established a genetic association between preeclamptic mothers and the *FLT1* (Fms related tyrosine kinase 1) gene.
- The same variants may also protect Finnish women from heart failure in later life.

What Is Relevant?

- Preeclampsia is a common hypertensive disorder of the pregnancy.
- Identification of protective variants in *FLT1*, a known candidate gene, opens an opportunity for drug development to target the women most at risk of the disease.

Summary

Genetic variants within the maternal *FLT1* protect Finnish women from preeclampsia.

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PROTECTIVE LOW FREQUENCY VARIANTS FOR PREECLAMPSIA IN THE *FLT1* GENE IN THE FINNISH POPULATION

Online supplement

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Table S1. Clinical characteristics of targeted exome sequencing participants*.

Characteristic	PE women (n=487)†	Controls (n=187)	P-value‡
Age, years; median (25, 75 percentiles)	29 (26, 32)	29 (26, 32)	0.993
Body mass index before pregnancy; median (25, 27 percentiles)	22.6 (20.9, 24.8)	22.5 (20.6, 25)	0.450
Proteinuria, g/l; median (25, 75 percentiles)	3.31 (1.59, 6.04)	na	na
Blood pressure: systolic, mmHg; median (25, 75 percentiles)	164 (152, 177)	125 (118, 133)	<0.001
Blood pressure: diastolic, mmHg; median (25, 75 percentiles)	108 (103, 114)	82 (78, 88)	<0.001
Gestation weeks; median (25, 75 percentiles)	38 (35, 39)	40 (39, 41)	<0.001
Mode of birth (vaginal deliveries n; %)	279; 57.3	160; 85.6	<0.001
Baby sex (% female)	52.9	49.7	0.450
Baby weight; g median (25, 75 percentiles)	2820 (2210, 3270)	3540 (3260, 3850)	0.119
Baby weight; 5% percentile cut-off value; g, n	1132, 24	2905.4, 9	
Placenta weight, g; median (25, 75 percentiles)	500 (405, 600) n=464	590 (500, 670) n=181	0.139

Pre-pregnancy smoking %	21.2, n=467	19.7, n=177	0.582
First gravidity (n; %)	328; 67.4	144; 77	0.030
Primipara (n; %)	450; 92.4	187; 100	0.005
Region of origin: Helsinki region (n; %)	251; 51.5	100; 53.5	0.652
Region of origin: Eastern Finland (n; %)	67; 13.8	23; 12.3	0.618
Region of origin: Northern Finland (n; %)	83; 17	29; 15.5	0.632
Region of origin: Central Finland (n; %)	39; 8	22; 11.2	0.188
Region of origin: Southwestern Finland (n; %)	47; 9.7	14; 7.4	0.381

* 97.4% of 500 women with PE and 98.4% of 190 control women passed quality control and their data is reported here and in the association analyses.

† 15.6 % of the preeclamptic women gave birth <34 weeks of gestation and 72.7% had severe preeclampsia

‡ Chi^2

BMI = mass (kg) / $height^2$ (m); na, not available (no proteinuria in controls).

Table S2. The candidate genes and intronic or near-gene loci

Candidate genes					Candidate loci	
					Gene	SNP
ACE	AGTR1	ERAP2	JAG1	ROCK2	AGT	rs699
ACVR1	AGTR2	ESRRG	KDR	SOD1	AGT	rs4762
ACVR1B	ANGPT1	F13A1	KIAA1239	SOD2	AGTR1	rs5186
ACVR1C	ANGPT2	FLT1	KIAA1462	STOX1	APOE	rs429358
ACVR2A	ANGPT3	FLT4	LCT	STOX2	CTLA4	rs231775
ACVR2B	ANGPT4	FN1	LIPA	SWAP70	ESRRG	rs17686866
ACVRL1	ANTXR1	GPR98	LMCD1	TGFB1	IFLTD1	rs10743565
ADAM10	Cdkn1c	HEY1	LPL	TGFB2	IL10	rs1800896
ADAM12	COMMD7	HEY2	LRRFIP1	TGFB3	KIAA1239	rs1426409
ADAM15	COMT	IFLTD1	MAGI1	TGFBR1	LMCD1	rs9831647
ADAM17	CORIN	IL10	MME	TGFBR2	LPL	rs1800590
ADAM19	CTLA4	INHA	NODAL	TGFBRAP1	LPL	rs268
ADAM28	DEF6	INHBA	NOS3	TIE1	near IL-10	rs1800871
ADAM8	DGKE	INHBB	NOTCH2	TIE2	near IL-10	rs1800896
ADAM9	EDN1	INHBC	PDGFD	TNF	NOS3	rs61722009
ADAMTS7	EDN2	INHBE	PDXDC1	VEGFA	NOS3	rs2070744
ADM	EHD3	IP6K1	PGF	VEGFB	NOS3	rs1799983
ADM2	EHD4	ITGA2	PSG11	VEGFC	VEGFA	rs3025039
AGT	ENG	ITGB1	ROCK1			